ON THE INADEQUACY OF THE CONCEPTION OF THE NEURONE AS THE UNIT OF CONDUCTION OF THE NERVOUS SYSTEM

by

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I propose in this paper to discuss certain aspects of the physiology of the nervous system, which, while they have previously been upheld by Apáthy and Bethe from purely morphological evidence, have failed to gain acceptance by physiologists; the question, namely, whether a single nerve *fibre* is capable of transmitting several impulses simultaneously. The evidence hitherto adduced in favour of this conception must be regarded as inadequate; in particular, in view of the clearly established "all-or-nothing" principle for the contraction of a muscle fibre when functioning under constant mechanical conditions, no good reason has been shown why a single efferent neurone should be capable of transmitting many impulses simultaneously, rather than one, to a muscle-fibre, when that fibre, as we know, is incapable of responding to the stimulus by graded intensity of contraction.

Our present knowledge of the structure of a motor end-plate, and its relation to the two excitable membranes of the muscle fibres, sheds an unexpected light on this question; and I believe that the relation of the end-plate to the muscle fibre becomes intelligible to us only on the assumption of the existence of numerous conducting paths within each motor neurone. During recent years, moreover, a number of observations have been made on the physiology of the sense organs and of reflexes, which seem explicable only on the view that the single neurone must contain numerous subsidiary conducting channels within it; and in the present paper I shall try to establish the following points:

1. The afferent neurone from a peripheral receptive cell responds by graded activity to graded strength of excitation of the receptor; and this graded activity of the afferent neurone is manifested, not by gradation in the frequency with which impulses pass along it, but by a gradation in the *size* of the disturbances propagated.

2. Since, however, the physical process of conduction is one of "all-or-none" intensity, it will follow that a gradation in the size of the disturbance propagated

must have as its basis the excitation of a graded number of conducting paths within each neurone. This will render it highly probable that the neurofibrils, which can be shown not to be "fixation artefacts," are the actual conducting paths.

3. Increase of reflex muscular response to increase in intensity of receptor stimulation depends upon the excitation of a larger number of efferent motor neurones (cf. Sherrington) (26), in addition, of course, to the summational effects of tetanus. The neurofibrils in the efferent neurones serve possibly as efferent paths for various reflexes which involve the same effectors, but are elicited from different receptors; it seems also as if antagonistic reflexes which employ the same effectors may excite them through different neurofibrils of the motor neurones. This will involve a partial modification of the principle of the Final Common Path.

General Remarks on Neurofibrils.

The discovery of neurofibrils we owe to Max Schultze; the theory that they, rather than the nerve fibres, are the actual conducting elements of the nervous system, originated in the work, also of Schultze, but especially of Apáthy (3) and of Bethe (6), who by inventing special staining methods have been able to demonstrate that the neurofibrils form continuous, unbroken paths within the neurones, showing, apparently, no anastomosis with one another, and having the essential property, therefore, of independent conducting elements. Bethe has sought to demonstrate that they are the only *continuous* structures within the fibres, the perifibrillar substance being discontinuous at the nodes of Ranvier; this would be strong, though not conclusive, evidence that the conduction takes place within the fibrils, but it cannot be said that his demonstration has been satisfactory. When one observes, as Bethe has done, that nerves which have been fixed in osmic acid, and especially fibres which have previously been stretched, are devoid of perifibrillar fluid at the nodes, it is clearly impossible for us to conclude whether this represents the real conditions within the living fibre, or whether it is a post mortem shrinkage effect. We cannot even positively assert that a continuous neuroplasm is essential for conduction; on the contrary, the conception of the accumulation of ions on semi-permeable membranes plays a large part in present-day theories of protoplasmic excitation. The second line of evidence introduced by Bethe, though strongly suggestive, is still inconclusive. It rests upon his observation that if a small region of a nerve fibre be compressed, then the capacity of the fibre to conduct impulses ceases at the moment when the neurofibrils lose their primary staining reaction (primäre Färbbarkeit). But if we assume, as many do, that the process of conduction involves an interaction between a membrane (fibril) and its surrounding

fluid, then clearly, cessation of conduction on disorganization of the neurofibrils can throw but little light upon the question, whether a single impulse of unalterable intensity is travelling through the neurone in the perifibrillar fluid, or whether an impulse is travelling in one or more of the fibrillae, thus permitting of a gradation in the intensity of the activity of the neurone, though not, of course, of its individual fibrils. The further observation of Bethe (p. 261), that a nerve fibre can still conduct with undiminished strength, even when compression has caused an almost total obliteration of perifibrillar substance from the region of compression, has little meaning at the present day in view of the discovery by Adrian (1) that a propagated disturbance, after undergoing almost total obliteration in a region of decrement, may immediately regain its maximal intensity on re-entering normal nerve. We must conclude, therefore, that while the evidence produced by Bethe is very suggestive, it has failed to demonstrate that the individual neurofibril, rather than the whole neurone, is the elementary conducting unit of the nervous system.

These observations of Apáthy and Bethe have encountered the most serious charge that can be laid against a histological observation—that of artefact. Basing their conclusions on the ultramicroscopic examination of isolated living nerve cells, bathed in serum or cerebro-spinal fluid, Mott (22) and Marinesco (20) conclude that the neurofibrils do not exist as such in the nerve cells during life, but that they are products of the coagulation of the cell cytoplasm, formed subsequent to the death of the cell. This view is accepted in Bayliss' "Principles of General Physiology." It seems to me, however, that this conclusion is entirely unjustified; microscopic examination of living cells can reveal the presence of structures within the cell-body only when the refractivity of these differs markedly from that of the remainder of the protoplasm. Anyone who has ever experienced how difficult or impossible it may be to detect so characteristic a structure as the nucleus in certain living individuals of amoeba, will attach but little weight to negative observations made upon living material. It may be noted, indeed, that in many of Marinesco's microphotographs nuclei are either indistinct or quite invisible. On the contrary, it is pointed out by H. Freundlich (12) that while the particles of certain colloids, such as gold, provided they are large enough, are readily visible under the ultramicroscope, "on the other hand, the particles of colloidal protein or starch solutions are generally invisible, since the solution molecules here contain so much water that their optical properties closely resemble those of the surrounding fluid. The ultramicroscope is therefore a powerful aid in the recognition of the colloidal nature of a solution, but we must not infer the absence of colloidal particles from a failure of the ultramicroscope to reveal them." We possess, on the other hand, good evidence that the neurofibrils are actual constituents of the cells. When structures, having an identical appearance, can be revealed by treatment of nerve cells by such diverse methods as fixation with strong formalin, or with dilute osmic acid, on the one hand, or by immersion

in exceedingly dilute methylene-blue, in which no real fixation can be said to occur, on the other, then we have very strong evidence that we are not dealing with artificial effects, but that the treatment is revealing structures that are present in the living cell. Further evidence in favour of this view will be given towards the end of this paper.

Others, notably Verworn and Goldschmidt, regard the neurofibrillae as skeletal elements, whose function it is to form a support for the fluid "neuroplasm" in which they lie. It is difficult to obtain any real evidence for or against this view. When we observe, as we can do so clearly in the nerve-nets of small blood-vessels, that a single neurofibril may break out from a nerve fibre and run its own course, it is impossible to state from mere inspection whether the nervous impulses travel in the neurofibril, or whether they are conducted by a minute amount of "neuroplasm" which may surround the fibril. Lenhossek (15) has justly dissented from this view of their function, on the ground that if they were merely skeletal elements, one would scarcely expect such an extraordinary array of fibrils within the nerve cells. It is indeed difficult to see in what way the neurofibrils can form supports for the neuroplasm of the nerve axons, when they lie within that neuroplasm, and run in a direction which is least suited to counteract any pressure that may be exerted upon the nerve. Their disposition within the fibres is such that if they were skeletal elements they should counteract tension; compare, for example, the fibrils sometimes seen in connective tissue. It is obvious, however, that such a rôle, if efficiently carried out, would prove disastrous to the nerve. Moreover, many nerves, such as the internuncial fibres within the brain, are never exposed to tension, yet they are exceedingly well supplied with neurofibrils. We must surely conclude that if, as various authors maintain, neurofibrils were skeletal elements, then they should be situated outside the neuroplasm, and not within it. If their function were to maintain an organization of the fluid or semifluid contents of the axon, one might expect to find a radial distribution of neurofibrils; but it would be difficult to see in what manner a *single* neurofibril, traversing a very bulky perifibrillar fluid, such as we find in the motor neurones of *Hirudo*—see Bethe's book (6), figs. 2 and 3—could in any way act as a support for the perifibrillar fluid.

Lenhossek (16) likewise attributes a skeletal supporting function to them, but believes that they exert this supporting function only during embryonic life. As the neuroblasts grow out from the embryonic nerve cord, they meet strong resistance from the tissue through which they grow; he believes that the neurofibrils develop, in order to impart a rigidity to the neuroblasts sufficient to enable them to overcome this resistance. Their persistence in the adult animal he explains largely on the ground that they may be required for regeneration purposes by the nerve, in case of injury. It is clear, however, that the theory of Lenhossek will not account for the occurence of neurofibrils within nerve cells which are devoid of long axons, such as the second type of Golgi cells.

While, then, each of these theories has something in favour of it, it seems that the evidence adduced in support of any one of them is inadequate; it is, indeed, almost impossible to form a conclusion on this point by mere microscopic examination of the fibrils. Nevertheless, I believe that there are a number of well established physiological observations which are quite incompatible with the view that the neurone is incapable of grading the intensity of its response. If this point can be proved, then, in view of the fact established by Verworn and his school, and by Adrian, that the physical process of conduction is, in normal nerve, one of "all-or-none" intensity, it will follow that underlying this gradation there must occur the excitation of a graded number of conducting paths within the individual neurones. If this can be proved, it will constitute very strong evidence that the neurofibrils are indeed the elementary conducting units, as Apáthy and Bethe have maintained.

It must be admitted, at the same time, that in ascribing a conducting function to the neurofibrils, a number of difficulties are encountered. In the well-known figures of ganglion cells prepared by the reduced silver method, and depicted by Cajal and others, the fibrils are usually not shown as isolated from one another, but, on the contrary, appear entangled with one another by a very delicate network which pervades the cell. But it is almost certain (4, 13) that we are dealing here with a fixation artefact, the various fibrils adhering together as the cell cytoplasm coagulates under the influence of the fixative. Bethe, on the contrary, is able to make preparations in which no such anastomosis occurs. This point is especially strongly insisted upon by Jäderholm (13), and by F. M. Ballantyne (4), who believe they can trace individual neurofibrils right through the ganglion cells, without their undergoing any extensive anastomosis with adjacent fibrils. I believe that this conclusion is only partly correct, and shall discuss it more fully at the end of this paper.

The same objections may be raised against observations in which anastomosis of neurofibrillae is described within the nerve fibre. Bethe, on the other hand, has observed them running entirely independent paths within the fibres. I believe I can confirm this observation, although it must be admitted that it is usually an almost impossible question to decide with certainty, so closely do they run together.

Histologists are almost unanimous in their opinion that in the terminal end-plates of motor neurones a true anastomosing network of neurofibrils occurs. The same criticism applies here also; moreover, Boeke (8) has been able to obtain preparations in which the fibrils proceed independently within the end-plate, and terminate each in a small loop; and he inclines to the view that no real anastomosis of fibrils occurs in this situation.

I have examined the neurofibrillae in the motor nerve endings, in the frog, and in contradiction of the work of Cajal, can entirely confirm Boeke's conclusion. By means of Bielchowsky preparations, I find that the neurofibrils, separating

out from one another in the terminal end-plate, run an entirely independent course, and end in little loops. The "loops" seem to be identical with the end-plate "knobs" referred to below. Two such nerve endings, taken from the frog's biceps muscle, are illustrated in figure 5. When we remember the great opportunity presented to the neurofibrillae for sticking together during the coagulation

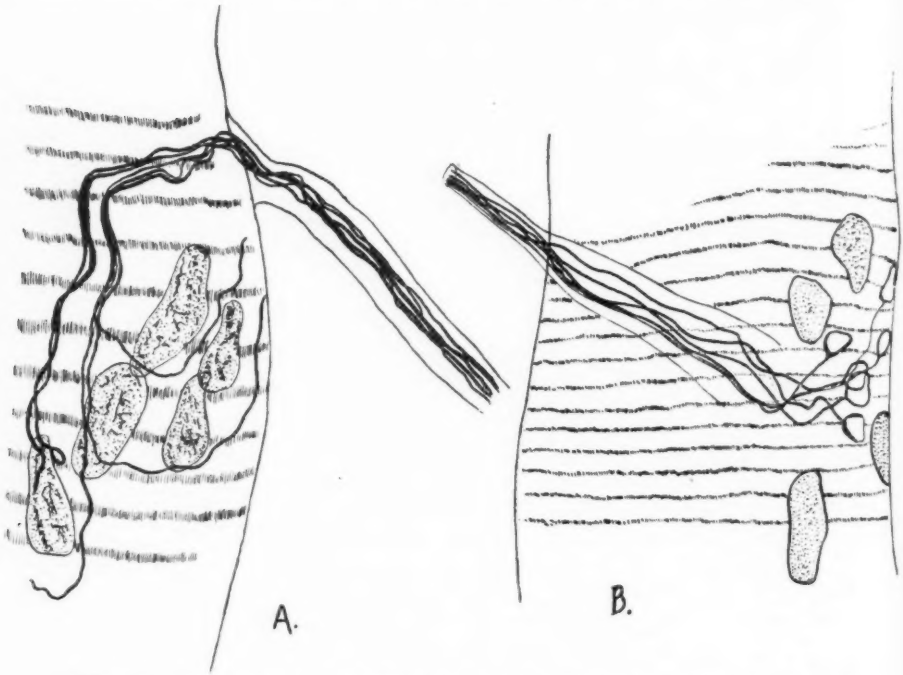


Figure 5. Distribution of neurofibrils in motor end-plate; from biceps muscle of frog (*Hyla aurea*).

A. Note that the neurofibrils show no tendency to form anastomosing networks. In this preparation only one end-knob has been revealed.

B. This specimen, like the last, is from a longitudinal section along the muscle fibre, and only part of the end-plate is seen in the section. Note, again, that the neurofibrils run a perfectly independent course within the end-plate, and also within the axon. Note also the neurofibrils ending in small loops ("end-knobs").

(Bielchowsky reduced silver method.)

of the fluid cytoplasm in which they lie, then we are justified, I believe, in laying greater stress upon preparations in which the neurofibrils run an independent course within the neurone.

A much more real difficulty lies in the fact that many neurofibrils within the ganglion cells do not appear ever to enter the axon, but travel, on the

contrary, from one dendrite to another. This would be a very serious objection. I do not believe, however, that the observation is as well established as the figures of Bethe and of Jäderholm may lead us to expect, and shall discuss this more fully below.

On Gradation in the Intensity of Sensory Excitation.

The vertebrate spinal cord is a structure of such extreme complexity that it is well nigh impossible from a study of its reactions to decide whether we must consider the neurone as the unit of conduction, or whether we must attribute a capacity for conduction to its constituent neurofibrils; when, for example, we observe extensive gradation of reflex response to a gradation of intensity of stimulation of a peripheral skin-point, we are not able to say definitely whether the afferent neurones have conducted disturbances of graded intensities, or whether central summation and facilitation alone can account for it. For this reason we are likely to derive much more definite results from a consideration of the behaviour of an organ of whose structure and mechanism we have a more precise knowledge. I believe that the human ear lends itself well to this purpose.

The cochlea may be considered as a strip of sensory epithelium, the excitation of any given hair-cell, or set of hair-cells, producing an auditory sensation of a particular definite pitch. The very important histological fact has been discovered by Cajal (9), by Kolmer (14), and by Bielchowsky and Brühl (7), that each hair-cell is innervated by a *single* nerve fibre, which is continuous with a single ganglion cell in the spiral ganglion. The nerve fibres form at the base of the hair-cell each a cup-like expansion, which can now be seen to be composed of numerous very delicate neurofibrils, which closely embrace it (Kolmer).

Of the two theories of hearing that have prevailed in the past, that of a peripheral and that of a central analysis of the sound, the former alone can be considered at the present day; the latter theory, especially in the form in which it was propounded by W. Rutherford (1886), is definitely contradicted by the fact that nerve fibres are unable to transmit impulses at the high frequency that it demands. Moreover, as has recently been shown by Wilkinson and Gray (32), contrary to the statement of Keith, the cochlea is a structure which is capable of responding to sound waves of different frequency and intensity, very much in the manner postulated in Helmholtz's theory of peripheral resonators. Assuming, then, the correctness of the theory that the analysis occurs peripherally, we can further state that the excitation of a certain receptive cell, or group of such cells, produces always a sensation of a note of particular pitch. The clearest evidence for this statement lies in the phenomenon of auditory fatigue, where the ear becomes fatigued to a sound of particular pitch to which it has been subjected, but remains unaffected for all other notes. Irrespective, then, of whether we adopt the resonance theory of Helmholtz, or the much less probable

sound-pattern theory of Ewald, we can conclude that sound waves of a particular frequency and amplitude excite in us a sensation of a note of particular pitch and loudness, by stimulating with a certain intensity a certain localized hair-cell, or group of hair-cells, in the cochlea. This is the view held by most physiologists; I have here merely repeated the facts on which it is based, in order to be more certain of the point which underlies the subsequent argument.

It has long been an obscure problem in the physiology of hearing how the central auditory mechanism can be apprised of the different degrees of loudness of sounds, when the auditory nerves are unable to conduct from the ear impulses of graded intensity. An ingenious explanation, based on the fact of the reduced excitability of nerves during the relatively refractory period, has been offered recently by Forbes and Gregg (10). I do not believe, however, for reasons that will be given below, that this explanation is an adequate one.

Underlying a gradation in intensity of sensory response, we may imagine three different processes: (1) There may occur an excitation of a varying number of afferent neurones; or (2) the impulses, whether of normal or of sub-normal intensity, may pass along the nerve at graded frequency; or (3) the disturbance propagated within the single neurone may be of graded intensity.

1. Gray (32) has considered the first of these processes as playing a large part in the mechanism of the recognition of degrees of loudness. Taking into consideration the fact that, in tactile sensations, with increasing pressure the increase is judged as occurring only at the actual point of application of the object touched, although receptors over a considerable surrounding area must be excited (as seems to be proved by the blanching of the skin capillaries here), he points out that a similar phenomenon must occur within the ear, for since the vibrating fibres of the basilar membrane are loosely bound together, vibration of the fibre, which is in perfect resonance, will be accompanied by a partial spread of the vibration, and the extent of spread will be the greater, the greater the amplitude of swing of the maximally stimulated fibre. He suggests that a gradation in the number of fibres thus secondarily brought into action may serve as the basis for discrimination of intensity of auditory excitation. There are, however, several fatal objections to this view. In the first place the well-established fact of auditory fatigue proves beyond doubt that in hearing a very loud note, some *single* irritable cell, or group of cells, has been functioning with great activity; we must conclude that the basis of graded intensity of hearing is a graded activity of a single sensory unit, and not a gradation in the number of sensory units stimulated. A further serious objection lies in the fact that a large number of soft notes nearly but not quite in unison, should give rise to a sensation of a note of maximal intensity; we know, however, that they merely produce an unpleasant discord. A third objection is that the theory will obviously not apply to the eye. As a modification of this theory it might be

urged that the number of upper harmonics that attain threshold value might serve for the discrimination of loudness; it is well known, however, that they will affect merely the quality of the note. From these considerations, then, we must conclude that *gradation of loudness of hearing does not depend upon a variation in the number of auditory nerve fibres excited.*

2. A more obvious suggestion, in that it would permit of central summation effects, is that gradation in the *frequency of passage* of impulses along the auditory nerves, whether of normal or of subnormal intensity, might underlie gradation in the intensity of the sensation experienced.

Let us consider first the case of gradation of frequency of impulses of *normal* intensity. Suppose a pure note is sounded repeatedly at very short intervals and at low intensity; as we sound the note more and more rapidly it will eventually be heard as a continuous note, whose loudness we can increase by striking it with greater force. There are two reasons why the note may become a continuous one: it may be that the receptive cell is transmitting to the auditory centre the maximum number of impulses that the auditory nerves can carry, in which case increase of the loudness of the note cannot be due to any further increase in the frequency of passage of impulses of *normal* intensity along the nerve. On the other hand, we may assume that the note becomes continuous long before the auditory nerves are transmitting normal impulses at maximum frequency. Suppose that in this case we now sound the note twice as rapidly as is required to produce the sensation of a continuous note; the note should now sound twice as loud, and in the limit, any note which is sounded as a continuous, sustained note must be one of maximal intensity, which we know to be untrue. We must conclude that gradation in the intensity of hearing cannot have as its basis gradation of frequency of passage of *normal* impulses along the fibres of the auditory nerve.

A very ingenious suggestion has, however, recently been put forward by Forbes and Gregg (10) to explain gradation in the strength of sensory excitation. From the researches of Keith Lucas and E. D. Adrian (2, 18) we know that by using stimuli of intensity above normal threshold value, nerves can be forced to conduct impulses during the subnormal conduction phase; and by increasing the intensity of the rapidly repeated stimuli, larger and larger numbers of impulses, each of subnormal intensity, can be excited in the nerve per unit of time. Forbes and Gregg suggest that the receptive cell may act in the same manner as our artificial stimuli, and that the stronger the excitation of the receptor the larger the number of impulses that are imparted to the sensory nerves in a unit of time. They conclude that "if the sensory receptors provide to the afferent fibres sustained stimuli of graded intensity, the brain may be apprised of the intensity of the peripheral stimulus by the frequency with which the impulses come to the centre over the afferent nerve fibres."

Against this suggestion must be raised the objection, firstly, that if any

increase in the number of impulses thus reaching the auditory centres occurs, the individual impulses will be of subnormal intensity, and it is doubtful if any beneficial results will accrue by such impulses reaching the auditory centre; it is well known, for example, that if a strong stimulus be applied to the heart, while it is in the relatively refractory condition, the contraction that is elicited is of correspondingly diminished size. Furthermore, if we assume with Keith Lucas that the "synapses" represent regions of decrement, then the increased frequence of passage of subnormal impulses would lead to a condition of inhibition (Wedensky inhibition), rather than of increased central excitation.

Much more conclusive evidence against the suggestion of Forbes and Gregg is to be found in some remarkable observations by Lord Rayleigh (23), and by More and Fry (21), on the capacity of the human ear to recognize phase difference. In these experiments it was found that if two sounds of equal pitch and intensity, but having a difference in phase, were led, the one to one ear, the other to the other, then the sound was invariably judged as coming from that side which had the lead-in phase. If, as in the experiments of Rayleigh, the sound emitted by two tuning forks, tuned almost but not quite to the same pitch (the beat occurred once in about five to fifty seconds), were led off independently to the two ears, then when the lead of phase was on the right side, the sound was judged with unerring precision as coming from the right side; as the lead in phase gradually changed to the left side, the direction of the sound seemed as surely now to change with it. This phenomenon could only be observed with frequencies below about 700 d.v. per second.

In order to account for this extraordinary fact, Rayleigh was led to accept the "telephone" theory of Rutherford in preference to the resonance theory. Nevertheless, our present-day knowledge of the properties of a nerve fibre prevents us from adopting this conclusion. The real explanation doubtless lies on the lines already implied in Rayleigh's conclusion. When the lead of phase is on the right side, the condensation will attain threshold value on the right side slightly earlier than on the left; and we must assume that excitation of the homorhythmic hair-cell, and therefore of the corresponding auditory neurone, will occur slightly earlier on the right side than on the left. This conclusion is in no way invalidated by the fact that it requires considerably more than one vibration to excite the sense cells. Hence impulses will reach the auditory centre from the right side slightly in advance of those from the left, and this seems to be the basis on which judgment of direction is formed. But this carries with it the very important implication that *the frequency with which impulses pass along the auditory neurone from the receptive cell of the one ear, is equal to that with which they pass along the auditory neurone from the corresponding receptive cell of the other ear, provided the stimuli have been of equal intensity.* It might seem at first sight incredible that a living tissue should behave with such astonishing precision; nevertheless, the physical observation proves that such a thing does occur.

Suppose, now, a source of sound to be so situated, that the intensity of excitation of the two ears is unequal; and on the assumption that there will now occur a variation in the frequency of passage of impulses along the corresponding auditory neurones of the two sides, let us suppose that the source is so situated that the right ear (nearer) transmits 550 impulses per second to the central auditory mechanism, the left only 500. It is clear that at the end of every one-hundredth part of a second the lead of impulses reaching the auditory centre will change from one side to the other, consequently the sound would seem to come momentarily from one side, momentarily from the other, and no localization of the sound would occur. We know with certainty that this is not the case, and must therefore further conclude that *the frequency with which the impulses pass along the auditory nerves is independent of the intensity of the excitation.*

3. From these considerations, then, I believe we are justified in stating that neither the excitation of a graded number of afferent neurones, nor a gradation in the frequency of passage of impulses along the neurones, can serve as the basis for a discrimination of intensity of peripheral excitation; but that, on the contrary, it is the *size* of the disturbance propagated in each neurone that underlies this phenomenon. Nevertheless, we know that the actual process of conduction, if it occurs at all, is for normal nerve one of unalterable intensity; underlying a gradation in the size of the disturbance propagated in a single neurone there must occur, therefore, a gradation in the *number* of elementary conducting units excited within the neurone, and it does not seem unjustifiable to assume that these units are the neurofibrils. We reach our final conclusion, therefore, that *the number of neurofibrillae excited within a single auditory neurone serves as the basis of our judgment of the intensity of peripheral auditory excitation.* This may possibly throw some light upon the fact that a continuous increase in the intensity of peripheral excitation is accompanied by a discontinuous increase in the sensation (Weber's law).

The Physiology of Motor End-plates.

The end-plates by which motor neurones terminate upon skeletal muscle fibres reveal a number of features which appear to be incompatible with certain well-established principles of stimulation of excitable tissues, unless there exist numerous conducting paths within these end-plates. The long, branching end-plates of frog and snake muscle are especially instructive.

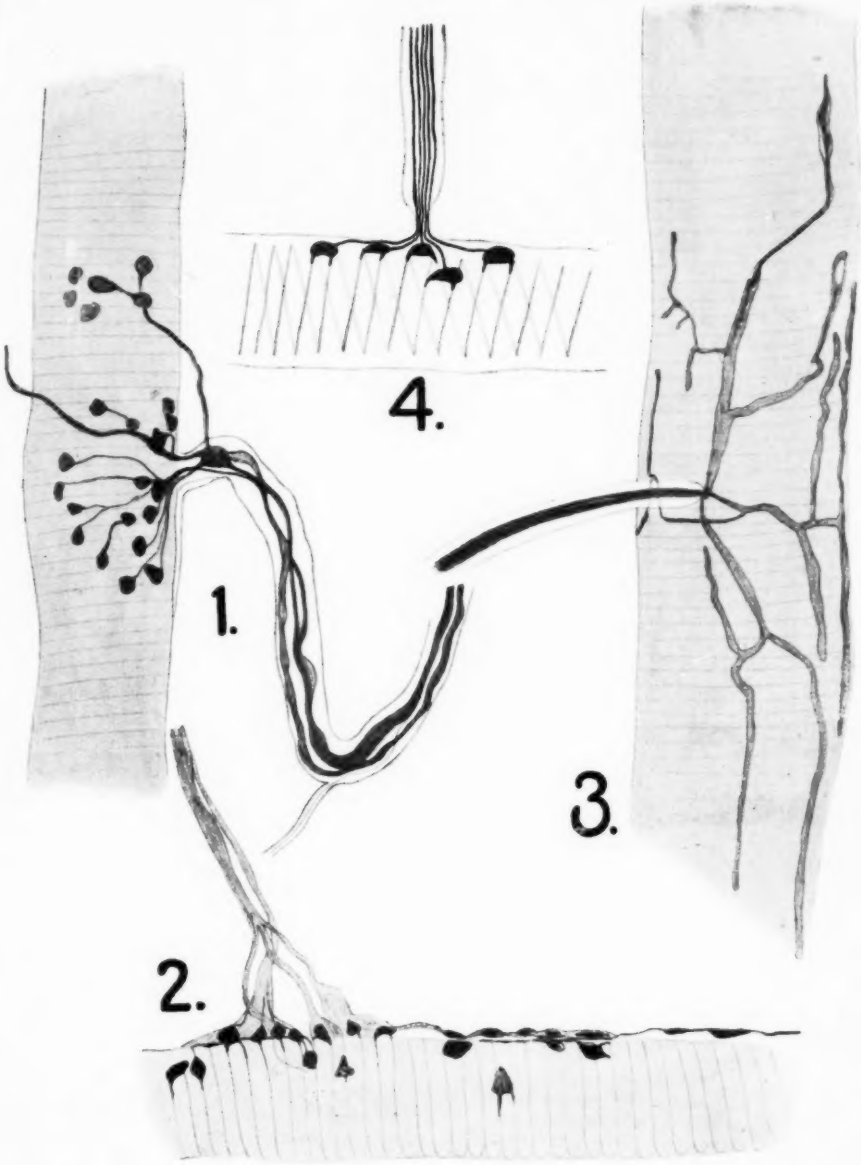
When we examine good preparations of these end-plates we often find that the axon of the nerve, immediately before entering the muscle fibre, divides into two or more parts, which then terminate separately upon the fibre. In figures 1 and 2, for example, are shown two end-plates, which have broken up into four parts just before penetrating the sarcolemma. The nerve endings in the sartorius muscle of the frog are usually formed by bifurcation of the axon, the two parts

then undergoing extensive branching on the fibre. In figure 3 is shown an end-plate, taken also from the frog's sartorius, which consists of three main branches. If we assume that the axon as a whole is the conducting unit, then it follows that the muscle fibre on excitation receives as many stimuli simultaneously as the motor nerve has terminal branches. But it seems to me that the histological observations allow us to go further than this.

In a previous paper (30) I have figured the nuclei[1] of the motor end-plate as connected directly with the membranes of Krause. These end-plate "nuclei" were also observed to be connected with very delicate fibrils, which could be traced for a short distance backwards into the terminal expansion of the motor nerve. The figure, which I have redrawn here (figure 2), shows a very important feature, whose meaning I did not at the time appreciate, namely, that many of the "nuclei" of the end-plate are connected, not with one, but with both the excitable membranes. The fact, firstly, that very delicate fibrils (which are probably the neurofibrils) can be observed to communicate with the end-plate "nuclei"; and, secondly, that these end-plate "nuclei" are connected, apparently, with both excitable membranes, seems to suggest that the excitation of a single neurofibril is sufficient to stimulate the two excitable membranes, and therefore to produce a single muscle twitch. In figure 1 is shown a motor end-plate from the mylohyoid muscle of the frog, in which the connection of the end-plate nuclei with very delicate fibrils (presumably neurofibrils) from the motor neurone, is seen. Concerning the connection of the end-plate "nuclei" with *both* excitable membranes, it must be admitted that this cannot always be observed; and, indeed, a considerable number of the "nuclei" shown in figure 2 are connected apparently only with one membrane. But we must remember that the methods at present available for demonstrating nerve endings are, from the cytological standpoint, crude, and possibly the connection of these nuclei with one membrane only may be due to shrinkage. It seems to me, therefore, that we have considerable evidence to show that the neurofibrils of the motor neurones part company terminally, and communicate each with an "end-knob," which then communicates often, perhaps always, with the *two* excitable membranes of the muscle fibre, as shown diagramatically in figure 4.

But whether we are willing to accept these finer histological details, with the implication that there are a large number of separate conducting paths to each muscle fibre, or whether we merely conclude that there are a small number of such terminal paths, according to the number of parts into which the motor neurone breaks up terminally, in either case we see that if the neurone is the unit of conduction of the nervous system, then the single muscle fibre will receive several stimuli simultaneously. Especially instructive figures will be found in

[1] The term which I have previously (30) used for these structures is misleading; they are quite different from the "sole-nuclei" of the end-plate, in that they are much smaller, and do not appear to contain chromatin. The term "end-knob" would be more suitable.



1. Motor nerve endings on muscle fibre (from mylohyoid of frog). Note the "end-knobs" connected by fine fibrils with the axon.
(Gold chloride preparation.)

2. The same, in side view. Most of the end-knobs are in direct connection with the excitable membranes; at least five of the end-knobs are in connection with both the excitable membranes.
(Gold chloride preparation.)

3. Motor nerve ending on fibre of frog's sartorius.
(Gold chloride preparation.)

4. Diagram to show the neurofibrlilar organization of a motor end-plate.

a paper by Sihler (24), who points out that in frogs and snakes the end-plate "sole" protoplasm, which is so characteristic of mammalian motor endings, is absent, and that excitation of the muscle fibre takes place through *numerous points of contact* between the nerve ending and the muscle fibre. What is the meaning of this curious fact?

It might be urged that the stimuli transmitted to the muscle fibre by the individual branches of the motor neurone are not strong enough to excite individually, but that several stimuli, each of subliminal intensity, reinforce one another, producing a stimulus which is now of adequate strength. But the researches of Keith Lucas and Adrian (17, 18) have shown that such a summation of inadequate stimuli can occur only if these stimuli are applied at the same point. Such a summation is believed to be a strictly localized, molecular effect, due, it is believed, to an accumulation of ions on the surface of a membrane, and the distance apart of even the finest branches of the motor end-plate far exceeds the range of action of molecular forces. If, on the other hand, we assume that the stimuli transmitted through the terminal branches of the motor neurone are above threshold value, then the end-plate is equally difficult to understand. The excitation wave from a stimulated point, situated anywhere along the fibre, spreads in both directions, from which we must conclude that one branch of the end-plate is sufficient to excite the muscle fibre, and all the others are superfluous. If there are more than two such terminal branches, then it can be shown that if the neurone is the unit of conduction, then all these branches, except the two outermost, can never play any part in the excitation of the muscle fibre, for the excitation to which they give rise will enter tissue left refractory by the passage of impulses from the two outermost branches, and so will be obliterated.

From these considerations we see, then, that a branching motor end-plate, or indeed even a continuous but spreading motor end-plate, applied to a series of successive points along a double helicoidal membrane (30), is a structure which in the light of our present knowledge of excitation is meaningless, unless there occur within it separate conducting channels, each independently excitable from the central nervous system. The histological observations are exceedingly difficult to make, for we do not at present possess an entirely satisfactory technique for revealing the finer details of motor end-plate organization; but so far as these observations go, they seem to show that the neurofibrils of the motor nerve are provided each with an "end-knob," and that these "end-knobs" are connected each with the two excitable membranes, so that excitation of a single efferent neurofibril will suffice to stimulate the entire muscle fibre.

From the above theoretical discussion it would appear that the motor neurone comprises not one, but numerous efferent paths to the muscle fibre, and that one only of these efferent paths is employed in a given reflex. A method is available for testing this deduction experimentally. If a motor neurone contains many efferent paths, one only of which is employed in a given reflex, then the current

of action for a motor nerve excited reflexly ought to be much smaller than for one stimulated directly with a maximum shock. It seems, indeed, that the theory of multiple efferent paths must stand or fall by this test. Unfortunately, most reflexes do not lend themselves for this experiment: firstly the reflex muscular response is usually very different from a single muscle twitch; secondly, the nerve trunk usually contains numerous motor fibres to other muscles which may not be involved in the reflex; thirdly, the nerve trunk contains both afferent and efferent nerve fibres, so that direct excitation of the nerve may excite many fibres which are not employed in the reflex. The flexion reflex in the hind limb of the cat to some extent avoids these difficulties; it may be elicited by application of a single shock to the afferent (popliteal) nerve. The reflex thus elicited in the decerebrate cat is nearly but not quite as high as the twitch obtained by direct stimulation of the motor nerve (Sherrington, 29). But it is considerably more prolonged, being a brief tetanus rather than a simple twitch. Furthermore, considerably less than half of the fibres in its nerve (peroneal) are afferent (Sherrington, 25).

Data concerning the question before us are available from the work of Forbes and Gregg (10), who have compared, in the decerebrate cat, the monophasic action current in the peroneal nerve, when excited directly and indirectly by maximal induction shocks. They find that the action current of the directly stimulated nerve is very much greater than that of the nerve when excited reflexly. The difference is in part to be accounted for as due to excitation, on direct stimulation, of the afferent neurones. On the other hand, the reflex contraction is not a simple twitch, but a tetanus of very brief duration, so that there will be a pronounced additive effect of the individual action currents, and this will not be diminished by the fact that the impulses in the whole nerve present the character of an overlapping series. The very great amount by which the reflex action current thus observed will exceed that due to a single reflexly elicited twitch (if such were obtainable) may be estimated by comparing the simple and the compound curves given by Forbes and Gregg in the latter part of their paper for summation in the current of action in muscle. For a discussion of the effect of *duration* of excitation, on the size of the galvanometer deflection, see J. F. Fulton (11). These quantitative measurements seem to show, then, that even if we allow for the excitation of the afferent peroneal neurones, *the directly excited action current is very much larger than the reflexly excited.*

Application to Spinal Reflexes.

When we attempt to apply these conclusions to the elucidation of the action of the central nervous system, serious difficulties are encountered. We are dealing here with a structure of such extraordinary complexity, that it is generally quite impossible to form a definite conclusion as to where a particular process is taking

place. When, for example, a facilitation or an inhibition has occurred, or when a prolonged reflex becomes fatigued, it is usually not possible to say with certainty to which part of a reflex are such a process must be ascribed. Nevertheless, certain facts are available on which it seems a general conclusion may be based.

In contradiction to the earlier statements of Wundt, Baglioni, and others, it has been found by Sherrington (27, 28), that many reflexes do not behave in an "all-or-none" manner, but that a very marked gradation in the size of the reflex response, with gradation in the intensity of the excitation may occur. Since this effect may be obtained by stimulation of *minute "skin-points,"* excitation of more or fewer afferent neurones seems to be excluded. If the conclusion reached above, concerning the gradation in the activity of auditory neurones, be applied to afferent neurones in general, then it seems that we may gain considerable insight into the nature of this gradation of reflex response.

When an afferent neurone enters the spinal cord it does not usually terminate upon a single nerve cell there, but on the contrary may continue a considerable distance up or down the cord, giving off collaterals to a very large number of nerve cells. According to independent observations by Bethe (6, p. 55) and Lenhossek (15), *single neurofibrillae separate out from the main bundle of neurofibrillae in the axon of the afferent fibre, and one neurofibril passes into each collateral.* Consequently, with gradation in the intensity of excitation, since there will occur, as we have seen, a gradation in the number of neurofibrillae excited in a single sensory neurone, therefore there will occur a corresponding gradation in the number of efferent fibres stimulated, with a corresponding gradation in the intensity of the muscular response. At the same time, we must not underestimate the effects of central facilitation and summation, in cases in which we are dealing with stimuli of longer duration than a single shock; it seems, nevertheless, that the breaking down of graded synaptic resistances will not entirely account for the gradation. For, if we assume, on the basis of our experience with the ear, that the sensory nerves do not respond by increased frequence of passage of impulses, to increased peripheral excitation, then it will be clear that central summation will not explain a gradation in the size of the *initial* contraction of the reflex response, although a subsequent increase in the response with continued application of the excitation will doubtless have central summation as its basis. Nevertheless, when extensive irradiation of impulses, in response to very intense excitation, occurs, it would be absurd to suggest that the effect would be due merely to excitation of the longest neurofibrillae.

From these considerations, then, we conclude that in response to an increase in strength of excitation of a receptor, more neurofibrils within a single afferent neurone are stimulated. These neurofibrils, separating out from the main trunks of the neurones into the collaterals, within the spinal cord, bring about excitation of more efferent neurones, with a resulting increase in the reflex response. There

is one objection which may be raised against this conclusion: if greater activity of the receptor brings about an excitation of more neurofibrillae within the neurone, may it not chance that some of the neurofibrillae, excited even by light stimulation, be those that pass to the more distant muscle groups? It is necessary to assume that such a thing does not occur, and that the longer neurofibrillae, perhaps on account of a greater excitation threshold, are excited only by the strong stimuli. The difficulty is encountered even when we regard the neurone as the unit of conduction, for in this case it would be necessary to assume a greater synaptic resistance for the more distant central branches of a sensory neurone.

The previous discussion on the motor end-plate has led to the conclusion that each muscle fibre is connected, not by one, but by numerous efferent paths with the central nervous system. This constitutes the greatest difficulty that the neurofibril theory has to encounter: we could readily understand the presence of numerous neurofibrils within sensory neurones, but it is not easy to see why so many efferent channels should exist, when apparently one alone would suffice for each muscle fibre.

Before discussing this point it will be useful to refer to one animal—the leech—in which this apparently primitive condition seems to have been realized. The researches of Apáthy and Bethe have show that in this animal the motor neurones possess each only a single thick neurofibril, while the sensory neurones possess a large number of smaller fibrils. At the periphery the fibrils of the sensory neurones part company, and are distributed to *numerous* skin receptors. When these sensory neurones are traced into the nerve cord the neurofibrillae are found to enter the central fibrillary mass (neuropil), where it is no longer possible to follow them individually. From this "neuropil" bundles of fibrillae again collect, and pass into the ganglion cells, where they form a "basket-work," from which a single thick neurofibril arises. This neurofibril passes outwards then in the motor neurone, which therefore has only a single neurofibril. So far as the connection of the sensory and motor neurones is concerned, it is necessary to assume that the fibrils of the bundle which enters a particular ganglion cell, are not identical with those of the corresponding sensory fibre; for under these circumstances it would be a simple matter to trace such a connection, and the neuropil would present the appearance of regular bundles of fibrils, rather than of an irregular, bizarre "Punktsubstanz." The actual morphological homologies of these structures are uncertain; this will, however, in no way affect the main argument.

The nervous system of the leech appears to illustrate in the most vivid manner the principle of gradation of reflex response, which appears from quite different considerations to prevail in the vertebrate nervous system also. Since the neurofibrils of the sensory neurones communicate peripherally with numerous skin receptors, it is scarcely possible to doubt that a gradation in sensory response

must occur. It will be a gradation, not indeed to intensity, but to extensity of excitation. It is clear, however, that for tactile stimuli extensity of excitation is, to some extent, a measure of intensity. The greater the peripheral excitation, therefore, the larger the number of neurofibrils in each afferent neurone excited. In the central neuropil a rearrangement of these fibrils occurs, resulting eventually in the excitation of more efferent neurones, i.e., in an increase in the muscular response.

But in the vertebrate, conditions are much more complicated, and the efferent roots contain numerous neurofibrillae. Perhaps this is to be correlated with the vastly greater variety of muscular movements of which these animals are capable; but we do not appear to possess enough data on which a generalization may as yet be based. Nevertheless, if we assume that not the neurone, but rather its constituent neurofibrillae are the actual conducting units of the nervous system, then a number of anomalies which seem to prevail in the physiology of reflexes, may find an explanation.

For example, it has been observed by Sherrington, that after a scratch reflex of a dog has been fatigued by exciting various points of the receptive field, the flexion reflex, which employs the same flexor muscles, can still be excited with ease. This leads to the conclusion that while most nerve cells are very rapidly fatigued, the final common neurone is relatively unfatiguable. Might not the anterior horn cells owe this characteristic to their possessing many instead of but one efferent conducting paths, the one conducting path being employed in one reflex, the other in another, perhaps antagonistic reflex, but involving the same effector? We should bear in mind the extraordinary observation of Sherrington (28), that a reflex, after being totally fatigued, may even under continued stimulation suddenly recover its normal strength, and repeat this several times. It seems impossible that the fatigue should have occurred at the first "synapse"; could it not have occurred at part of the final neurone, and a new path then become laid out, by summation, to another part of this final neurone? If we believe that relatively little fatigue occurs in the anterior horn cells, we should have to face the fact that histologically the most profound fatigue effects can be detected here (Gustav Mann, 19).

Consider the case of the displacement of a reflex by another reflex that employs the same effector, but to a different purpose, e.g., the displacement of the flexion reflex by the scratch reflex. If the terminal neurone is regarded as divisible, the inhibition of the flexion reflex might be conceived as occurring on part of this neurone, while the remainder of the neurone still remained responsive to stimuli from the afferent neurone which evoked the scratch reflex. But if we conceive the terminal neurone as indivisible, then we should have to place the inhibition at some more central internuncial neurone; and the conditions within the cord are so complex that we cannot positively deny this conclusion. But if the inhibition occurs here, rather than at the last motor neurone, then there

would clearly be no mechanism for the inhibition of after-discharge from the terminal motor neurone, and yet Sherrington's figure seems to show that the after-discharge characteristic of the flexion reflex is cut off very short when the scratch reflex replaces it—see Sherrington (28), figure 51. We should then have to conclude, either that no after-discharge occurred on the anterior horn cells, or, as an alternative, that these were indeed divisible. Nevertheless, recent work by Ballif, Fulton, and Liddell, (5) seems to show that an after-discharge from the anterior horn cells can indeed occur, and be inhibited. The experiment consists in the inhibition of the after-discharge of the knee-jerk, a reflex in which, it is usually believed, no internuncial neurone is involved.

But in those cases of inhibition in which we are not faced by the above dilemma, we usually locate the inhibition on the anterior horn cell, and the histological observation of Cajal (9), that a very large proportion of posterior root fibres terminate directly upon the anterior horn cells, lends strong support to this view.

The conditions within the cord are, however, so complex that beyond this it does not seem possible, from a study of its reactions, to obtain any critical evidence for or against the theory of the divisibility of the terminal motor neurone. Since the conclusions based on this evidence are as yet too vague, the following histological observations on the gray matter of the spinal cord supply much more decisive evidence.

Histological Observations on the Structure of the Gray Matter of the Spinal Cord.

The following observations on the gray matter of the spinal cord have been made by the use of the reduced silver method of Cajal, and especially by the Bielchowsky technique for revealing neurofibrils. Unborn or newly born rabbits and guinea-pigs were employed.

When good preparations of the spinal gray matter, especially those stained by the Bielchowsky method, are examined, there are to be observed in this gray matter the following structure: (*a*) nerve "cells," of which the anterior horn cells stain especially brilliantly; (*b*) neuroglia structures; in the preparations which I have employed only the nuclei of the neuroglia tissue have stained (see figure 6), so that no difficulty arises in confusing neuroglia threads with fine nerve fibres; (*c*) large nerve axons, which may be observed in small numbers traversing the gray matter; (*d*) enormous numbers of fine, sometimes extraordinarily fine, fibrils, which arise as collaterals either from the posterior root neurones or from the various descending neurones of the surrounding white matter. These fibrils run often independently, in an irregular manner, through the gray matter, but are more usually seen traversing it in smaller or larger bundles (see figure 6). Sometimes it is possible to trace these fibrils very long distances, but sooner or later they usually run out of the plane of the section.

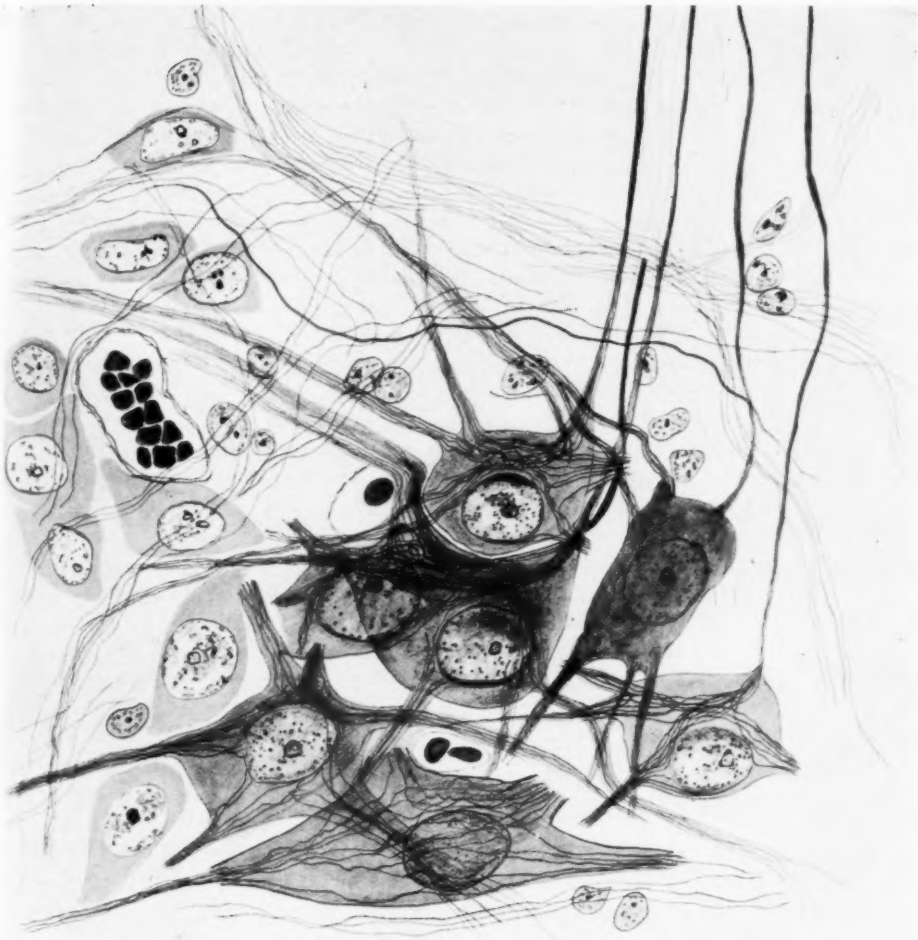


Figure 6. Group of anterior horn cells and surrounding grey matter from spinal cord of rabbit. In the region outside the nerve cells fine fibrils (collaterals) traverse the section, either in bundles or singly; they are frequently cut short as they pass out of the plane of the section. These fibrils can be traced, sometimes for long distances, into the dendrites of the nerve cells; details of the anastomosing system in the middle of the cell are not clearly recognizable in this example. Efferent neurofibrils then leave the cell by the darkly staining axon, five of which are seen in the section. In two of the cells the axon is out of the plane of the section; one of the nerve cells has over-stained, so that details of its neurofibril system are visible only in its dendrites. Numerous weakly staining nerve cells are seen at the left of the section; three blood-vessels are also present. The isolated nuclei are nuclei of neuroglia cells, whose cytoplasm has not stained.

(Bielchowsky reduced silver method.)

But large numbers of these fine fibrils can be traced, sometimes over short, sometimes over very long distances, to the dendrites of the anterior horn cells. At times, if the plane of section has been suitable, these bundles of neurofibrils can be traced back from the dendrites, and can be observed to arise by the convergence of fibrils from various parts of the gray matter (see figure 8, at x). At other times the bundles can be traced back from the dendrites over extremely long distances (see figure 6), but eventually pass out of the plane of the section, so that it is not possible to see whence they come. At other times, again, the fibrils which enter the dendrites (in the case of those cells which lie at the edge of the gray matter) can be traced outwards into the white matter (see figure 8).

In the preparations which I have examined, these observations can be made with absolute clearness, and at no time have I ever observed anything of the nature of a discontinuous synapse between these fibrils and the nerve cells. The fibrils penetrate the dendrites, and are now seen to be continuous with, and, indeed, quite indistinguishable from, the neurofibrillae of the nerve cell. No one would maintain that the fine fibrils that pervade the gray matter (outside the nerve cells) are fixation artefacts; it would seem equally erroneous to consider them as artefacts when they are once within the nerve cells. Furthermore, we must surely conclude that the fine fibrils that traverse the substance of the gray matter are conducting elements, probably the actual conducting units, since they arise as collaterals from the neurones that run longitudinally in the white matter. Ought we then to conclude that these fibrils, after they have entered the nerve cells, are no longer conducting elements, but are merely skeletal structures? It must be emphasized that these observations are in no way difficult to make, and provided well-stained material is employed, these facts can be observed with absolute clearness by the use of any high-power objective.

These neurofibrillae, on entering the nerve cell, run, in the dendrites, a perfectly independent course (see figures 6 and 7), and no fusion of neurofibrils can ever definitely be observed. But when the fibrils approach the middle of the cell, and sometimes even when still a considerable distance from it, they appear to branch, and the extraordinary difficulty which we now experience in trying to follow them individually is in marked contrast to the ease with which they may be observed in the outer portions of the nerve cells (see for example, figures 7 and 9). This is due to the convergence of the neurofibrillae in the region immediately around the nucleus, as they enter through the various dendrites, not only those in the plane of section of the nerve cell, but also through all the dendrites that are not in the plane of section. The difficulty is increased by the fact that we are generally observing the neurofibrillae on a very granular background, viz., the cell nucleus.

Despite these facts, however, I can state with considerable confidence that a true anastomosis of fibrils occurs round the nucleus. This observation is in agreement with the work of Cajal (see, for example, figure 75 of Cajal's book),

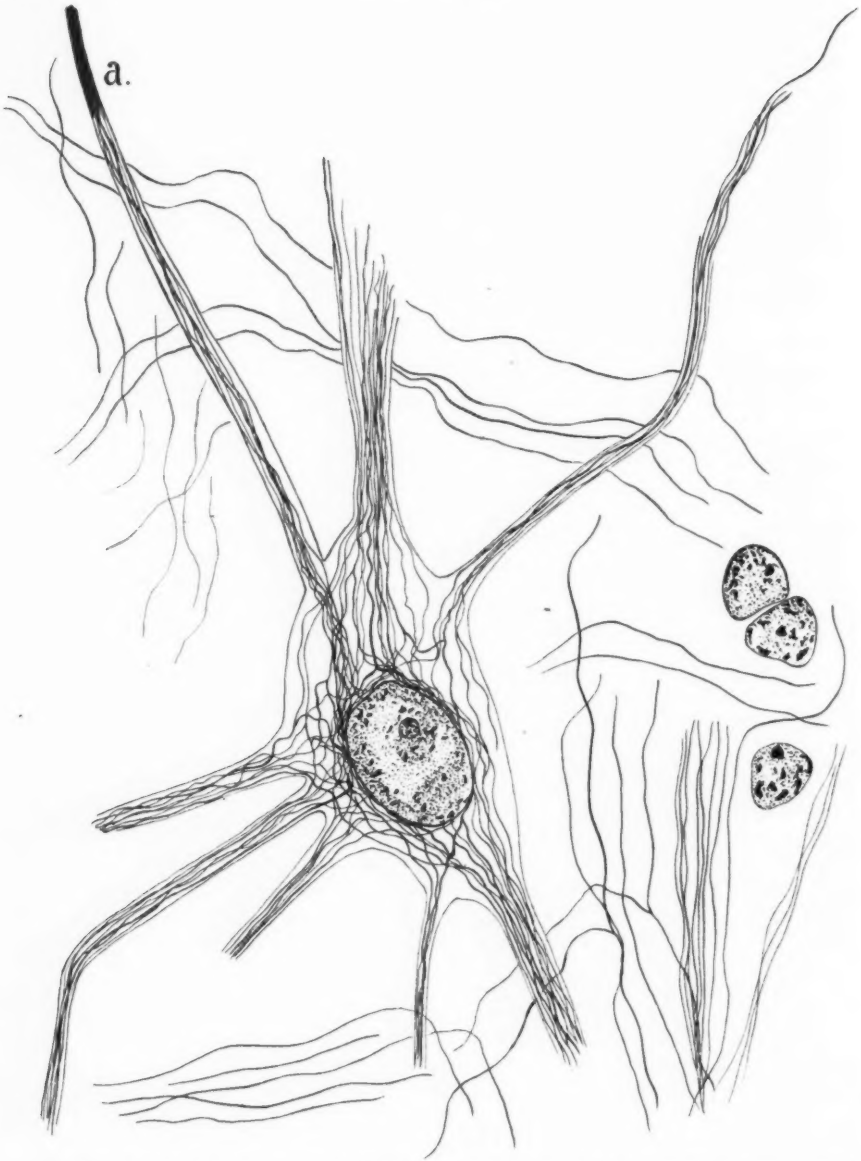


Figure 7. Anterior horn cell (rabbit), with highly developed neurofibril system. The section has been cut along the axon (a) and through the middle of the cell, so that the anastomosing system is not well seen; occasionally, however, some of the neurofibrillae show branching, even in the outer regions of the cell. Note, also, bundles of fibrils (collaterals) and the nuclei of three neuroglia cells in the surrounding extracellular region.
(Bielchowsky reduced silver method.)

and is opposed to that of Bethe. I do believe, however, that the fine meshwork often figured by Cajal as connecting up the whole fibril system is merely an artefact, due to faulty fixation of the cell protoplasm. I have certainly never seen anything resembling it in my preparations, and in this respect am wholly in agreement with Bethe.

The observation that the neurofibrilliae form an anastomosing system around the nucleus must, I believe, be regarded as well established; such a condition is seen, for example, in the nerve cell shown in figure 9, where the cell is viewed in a plane immediately above the nucleus (compare in this respect figures 7 and 9). Finally, from this anastomosing system a relatively small number of neurofibrils arise, and leave the cell by the axon.

These observations seem to throw light on the somewhat paradoxical observation of Bethe, that very many of the neurofibrillae enter the nerve cell by one dendrite and apparently leave it by another, without ever entering the axon; for if two neurofibrils, entering the cell by two dendrites, meet an anastomosing system, then clearly the appearance, especially if the section be a thin one, will be that of a single neurofibril traversing the cell from one dendrite to another. I have, indeed, often seen specimens having a close resemblance to those figured by Bethe, but have never been able to observe a case in which the above explanation could be definitely excluded. The two cells shown in figure 8 make this especially clear.

From these observations we conclude, then, that *the fine fibrils that arise as collaterals from the white matter, and pervade the whole of the gray matter, enter the dendrites of the nerve cells, either singly, or more usually in bundles, and without the formation of any discontinuous synapses (this observation has as yet only been made on the anterior horn cells), and that the fibrils (which are now spoken of as neurofibrils), entering the cells by the various dendrites, all converge towards the middle of the cell, where they undergo anastomosis; and that from this anastomosing system a relatively small number of neurofibrils arise and enter the axon.* No details can yet be given concerning the structure of the anastomosing network.

These latter remarks refer only, of course, to the anterior horn cells, so that the question of continuity or discontinuity at neurone junctions in other parts of the nervous system remains to be investigated further.

Concerning the mode of entry of the fibrils into the nerve cells, in my preparations I have never with certainty been able to observe these fibrils entering the *body* of the cell, as described by Bethe and by F. M. Ballantyne, while in every nerve cell that has stained in any way clearly they can be seen entering through the dendrites in very large numbers. I have also never seen anything resembling the Golgi network, described by Bethe as occurring on the outside of the nerve cells.

From these histological observations it seems, then, that we must conclude that the collaterals from the various neurones of the white matter enter the anterior horn cells without the formation of discontinuous synapses, and that the seat of the integration is in the anastomosing system in the deeper parts of the nerve cell. Sherrington's principle of "convergence" seems beautifully illustrated.

But since the neurofibril of the nerve cell, rather than the nerve cell as a whole, must, as we have seen above, be looked upon as the unit of conduction for the nervous impulse, so it seems we must also regard the efferent neurofibrillae that enter the axon as individual conducting units. But why so many efferent neurofibrils, rather than one only, as we find in the leech, should exist we cannot yet definitely state. Nevertheless, the thesis maintained in the foregoing theoretical discussion seems abundantly verified by the histological observations.

SUMMARY.

1. (*a*) From a consideration of the physiology of the organ of hearing, it is concluded that the auditory centre becomes apprised of a gradation in the intensity of the auditory excitation, not by a stimulation of a graded number of auditory neurones, nor yet by a gradation in the frequency of passage of impulses, whether of normal or of subnormal intensity, along these auditory neurones (for the ear is able to register phase difference), but by a gradation in the *magnitude* of the disturbance propagated in the individual neurones.

(*b*) But since the actual process of conduction is one of "all-or-none" intensity, it follows that, underlying this gradation in the magnitude of the response in each neurone, there must occur a gradation in the number of certain elementary conducting paths excited within each neurone.

(*c*) It is then shown that the neurofibrillae are not mere fixation artefacts, for they are capable of being revealed by wholly different methods of technique,

REFERENCE TO PLATE.

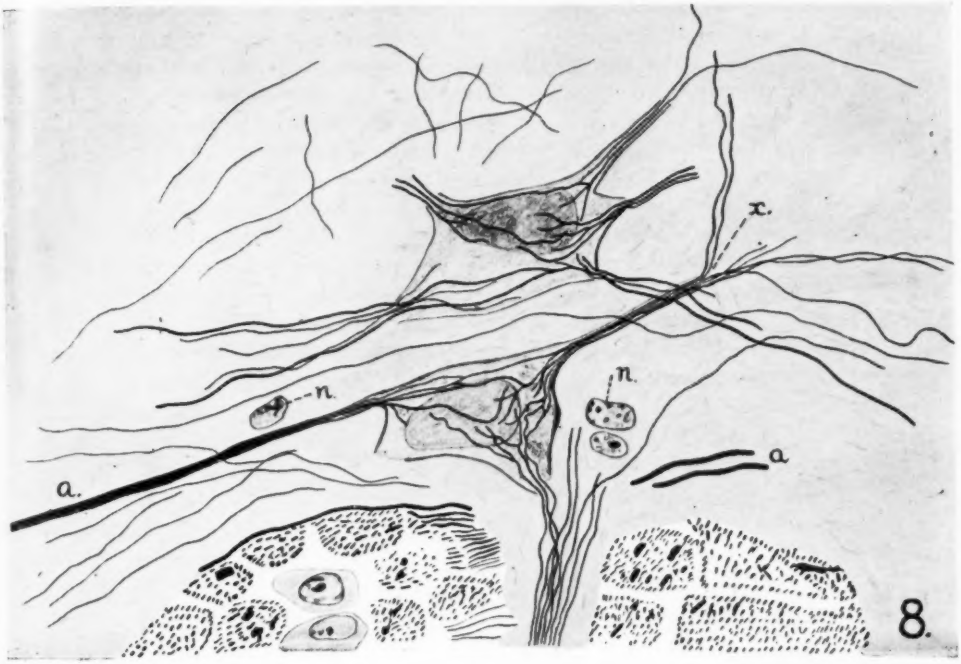
Figure 8. Two anterior horn cells from spinal cord of rabbit, taken from part of the grey matter, closely adjacent to the white matter. In the lower portion of the figure the longitudinally running bundles of nerve fibres of the white matter are seen cut in transverse section. A bundle of fine collaterals is seen, passing from the white matter into the grey; three of these collaterals enter one of the dendrites of the lower anterior horn cell; the others penetrate further into the grey matter, and soon pass out of the plane of the section. Portions of other collaterals are seen running irregularly in the grey matter. Within the nerve cells an indication of the anastomosing system is seen. At x, six fibrils are seen, converging from different regions of the grey matter, to enter a single dendrite.

a, axons; b, nuclei of neuroglia cells.

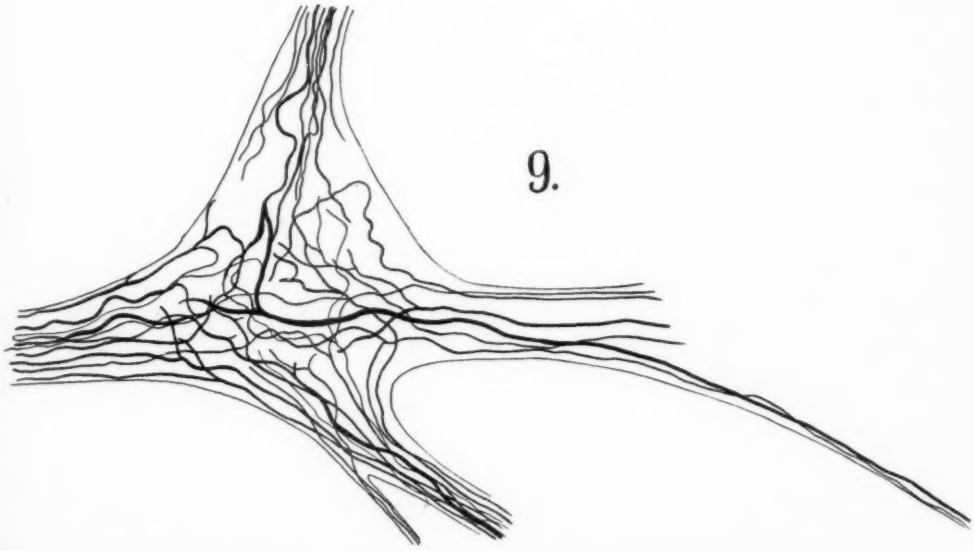
(Bielchowsky preparation.)

Figure 9. Large nerve cell from anterior horn of spinal cord (rabbit). The section is taken immediately to the side of the nucleus. The anastomosing neurofibril system is clearly seen.

(Bielchowsky preparation.)



8.



9.

and it is believed that the neurofibrils are the actual conducting units of the neurone. The conclusion is then reached, that the *receptive cells of the cochlea apprise the brain of the intensity of the auditory excitation, by grading the number of neurofibrillae they excite within the individual neurones.*

2. (*a*) From a consideration of motor end-plates as being branched and spreading structures applied in a discontinuous manner to a double helicoidal excitable membrane, it is shown that the absence of secondary conducting paths within these motor end-plates is incompatible with certain accepted laws of tissue excitation. Histologically it is found that the neurofibrils run entirely independent paths within the motor neurone, and that they terminate upon minute "end-knobs," which are in connection with the excitable membranes of the muscle fibre, frequently, perhaps always, with the *two* membranes.

(*b*) It is concluded that reflex excitation of a muscle fibre could be brought about adequately by the stimulation of a single neurofibril in the motor neurone. This is in agreement with the observation, that the directly excited action current of the motor neurone is very much greater than the reflexly excited.

3. Within the spinal cord the neurofibrillae of the sensory nerves separate to form each a collateral, one single afferent neurone thus coming into communication with numerous efferent neurones in the grey matter. This arrangement appears to provide the basis for the gradation of size of reflex response, to gradation in the intensity of stimulation of a peripheral skin-point, in those cases in which central summation seems unable to produce it.

4. The rôle of the many neurofibrils, rather than a single one, in the motor nerves is discussed. On the basis of certain phenomena of reflex action, the suggestion is offered that different types of reflexes which employ the same effectors, but to varying purposes, may act through wholly different final paths, and it seems as if the individual neurofibril may supply such a path. It is pointed out that the apparently primitive arrangement of the neurofibrillae is realized in the leech, whose sensory neurones contain many neurofibrillae, while the motor neurones contain but one.

5. In confirmation of these theoretical conclusions, it is shown histologically that the fine collaterals from the sensory and internuncial neurones traverse the grey matter, and eventually enter the dendrites of the anterior horn cells without the formation of synapses. The various collaterals, entering the anterior horn cells by its numerous dendrites, converge towards the middle of the cell, where they form an anastomosis; it is apparently here that the integration takes place. From this anastomosing system a small number of efferent neurofibrillae arise and enter the axon. In the axon they run an independent course, and eventually terminate individually upon the two excitable membranes of the muscle fibre.

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THE STRUCTURE OF THE NEURONE JUNCTIONS OF THE SPINAL CORD

by

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(Submitted for publication 7th May, 1926.)

In the previous paper I have tried to show that the conception of the neurone as the nervous conducting unit is inadequate to explain certain features of the behaviour of the central nervous system, but that such behaviour could be accounted for if we assume the existence of numerous conducting paths within the individual neurones; and it was pointed out that these ultimate conducting units were probably constituted by the neurofibrils. In confirmation of this view it was found histologically that the fine collaterals that traverse the grey matter in the spinal cord enter the dendrites of the anterior horn cells, either singly or in bundles, and become continuous with, and are indeed indistinguishable from, the neurofibrillae within the nerve cell, so that, since the fine collaterals were undoubtedly conducting elements, a conducting function would likewise have to be ascribed to them when they had penetrated the body of the nerve cell, i.e., when they had become identical with the neurofibrils of histologists. It was further pointed out that on the anterior horn cells, synapses, in the form in which they are usually conceived by physiologists, i.e., ramifying, brush-like terminations of neurones, coming into close contiguity, but not into direct continuity with the next neurone of the reflex chain, do not exist. On the contrary, the fine collaterals penetrate the nerve cells, and being now one and the same structure as the neurofibrils, run an independent course in the dendrites till they reach the middle of the cell. Here they appear to undergo anastomosis with the neurofibrillae that enter the nerve cell through its other dendrites, and this central anastomosing system appears to be the seat of the nervous integration.

These observations were obtained by the use mainly of preparations made by the Bielchowsky reduced silver method, and were confined entirely to the anterior horn cells; the posterior horn cells do not usually stain so sharply by this method. Now it has been shown by Sherrington (7) that, assuming the neurone to be the conducting unit of the nervous system, the anterior horn cells are characterized by certain features which distinguish them from all other spinal neurones; they are almost unfatiguable, and their discharge is practically

aperiodic. Since fatiguability and periodicity of discharge are regarded as features of synapse action, a simple explanation for this anomalous behaviour of the anterior horn cells might have been forthcoming if discontinuous synapses could have been demonstrated on the posterior horn neurones, or the neurones of Clark's column. But if we assume the terminal neurones to contain many conducting paths, it is unnecessary, as I have pointed out in the previous paper, to ascribe these peculiar properties to the anterior horn cells. The object of the present investigation was to find if any difference could be detected between the termination of neurones on the anterior horn cells, and those of the posterior horn, and of Clark's column. The result of this investigation has been to show that, so far as the microscope can reveal, *the mode of termination of neurones on all the spinal cord cells is the same, and no such thing as a ramifying, discontinuous synapse exists.* It is a curious fact that although numerous descriptions of fibre paths within the spinal cord are to be found in the literature of the subject, a true synapse has rarely been described; only one convincing illustration, so far as I know, has ever been given, namely, a case of several collaterals terminating on three nerve cells of Clark's column (see Cajal (3), vol. I, p. 311, fig. 110).

Methods.

For investigating the neurone junctions of the spinal cord I have used mainly material from very young rabbits (preferably unborn rabbits), though frogs, guinea-pigs, and cats several weeks old have also been employed. In some cases the tissue was stained by the Bielchowsky technique, but the reduced silver method of Cajal (using alcohol as a fixative) is the best process for revealing the structure of the posterior horn cells.

As much of our evidence for the existence of discontinuous synapses rests upon preparations made by the Golgi process, I have taken special pains to verify the results obtained by the reduced silver methods on material prepared by this latter process. I find that the Golgi method, while undoubtedly of inestimable value for tracing out fibre tracts, is entirely useless for revealing the finer details of neurone structure. As a cytological process it is valueless; it causes marked shrinkage of cells, fine precipitates may be deposited irregularly on the fibres and minute fibrils, and of the internal structure of the nerve cell it reveals nothing. While, therefore, the method must always be used as a valuable check on results obtained by the reduced silver process, the latter method must carry much greater weight when minute cytological details are in question.

Since the only convincing figure of a synapse (Cajal's figure) has been drawn from a preparation made by Ehrlich's methylene blue technique, much time has been spent in attempting to verify this observation of Cajal. I find, however, that methylene blue gives precisely the same results as does the Golgi

process, and no ramifying, brush-like terminations of fibres embracing the nerve cells can be revealed even by this method. At the same time, methylene blue preparations, made as they are without the aid of any fixatives, present numerous obvious artefacts when microscopically examined. The intercellular portions of the cord tend to become vacuolated, and the nerve cells themselves may at times present a frothy appearance. Obvious coagulation may often be seen on the surface of the nerve cells, and various intermediate stages between such frothing or coagulation, and the apparent ramifying synapses of Cajal may be observed. I find, too, that the superficial Golgi network described by Bethe as occurring on the surface of the nerve cells is to be looked upon as a result of coagulation, for all intermediate stages between such a network and undoubted surface coagulations may readily be observed.

I shall first describe the structure of the neurone junctions of the spinal cord as revealed by the reduced silver methods. Of the two processes usually employed, that due to Cajal and that due to Bielchowsky, the former has been, in my hands, more consistently satisfactory, though the Bielchowsky method, probably on account of its superior mode of fixation, is usually more successful in revealing the finer cytological details.

Anterior Horn Cells.

With the use of the Bielchowsky technique, and employing newborn rabbits and guinea-pigs, I find, as described in the previous paper, that the fine collaterals that arise from the longitudinal fibres of the white matter traverse the grey matter, sometimes in bundles, at other times irregularly alone, and eventually converge from various portions of the cord upon the dendrites of the anterior horn cells, and then penetrate them. Having penetrated the dendrites, they travel along them as the neurofibrils of the cell, and approaching the nuclear region of the cell, undergo anastomosis here. From this anastomosing system a smaller number of neurofibrils arise, which then emerge as the efferent neurofibrillae of the axon. The direct continuity of the neurofibrils of the nerve cell, with the fibrils that constitute the collaterals in the portion of the grey matter outside the nerve cells, seem to be sufficient proof that neurofibrils are not mere fixation artefacts, as maintained by many authors.

Various investigators (Cajal, Held, van Gehucten, Lenhossek, and others) regard the neurofibrils as merely a part of the protoplasmic reticulum of the cell. But as F. M. Ballantyne (1) has pointed out, the explanation is inadequate, for the reticulum may be observed between the fibrils.

Leydig (5), on the other hand, believes that they have a definite existence, but that, nevertheless, the real conducting element of the nerve cell is constituted by the interfibrillar substance. But the necessity for assuming the existence of separate paths within the neurones (8), and the fact that the neurofibrils are

continuous with structures outside the cell that are undoubtedly conducting elements, can leave little doubt that the neurofibrillae themselves constitute the conducting elements.

The Bielchowsky process seems, therefore, to show that in the anterior horn cells there is direct neurone continuity between these cells and the fibrils that terminate upon them. In the large number of cases that I have examined such neurone junction always occurs through the dendrites (cat, guinea-pig, rabbit). In these preparations I have never seen anything in the slightest degree resembling branching terminations of neurones ending upon the "body" of the nerve cells; the collaterals *all* enter the dendrites and penetrate to the middle of the cell. I have also never been able to observe the fine collaterals entering the Golgi network on the surface of the nerve cell as described by Bethe (2); while my preparations show neurone continuity with the utmost clearness, such continuity does not occur in the manner described by Bethe. F. M. Ballantyne has observed the neurofibrils directly penetrating the *body* of the nerve cell in *Lepidosiren*. But in the cat, guinea-pig, and rabbit I have never found such a thing to occur; the collaterals all enter through the dendrites. Neurone continuity within the central nervous system has also been described by Marui (6), cited from F. M. Ballantyne (1).

If the anterior horn cells are now examined in preparations stained by the reduced silver process of Cajal, it is frequently found, especially in strongly stained material, that the bundles of fine collaterals that penetrate the dendrites stain a deep black, and it may be difficult, at times impossible, to reveal neurofibrils within them. Nevertheless, even in such preparations, the collaterals can often be seen converging singly upon the dendrites, though it is not possible usually to follow out the individual neurofibrils once they have entered the dendrites, so heavily do the latter stain. But in such preparations, provided that the staining has not been too heavy, it is usually possible to observe neurofibrils again at the point where the dendrites merge into the "body" of the nerve cell. It would seem that these Cajal preparations throw a considerable light upon the misconstruction that has been placed by various investigators upon

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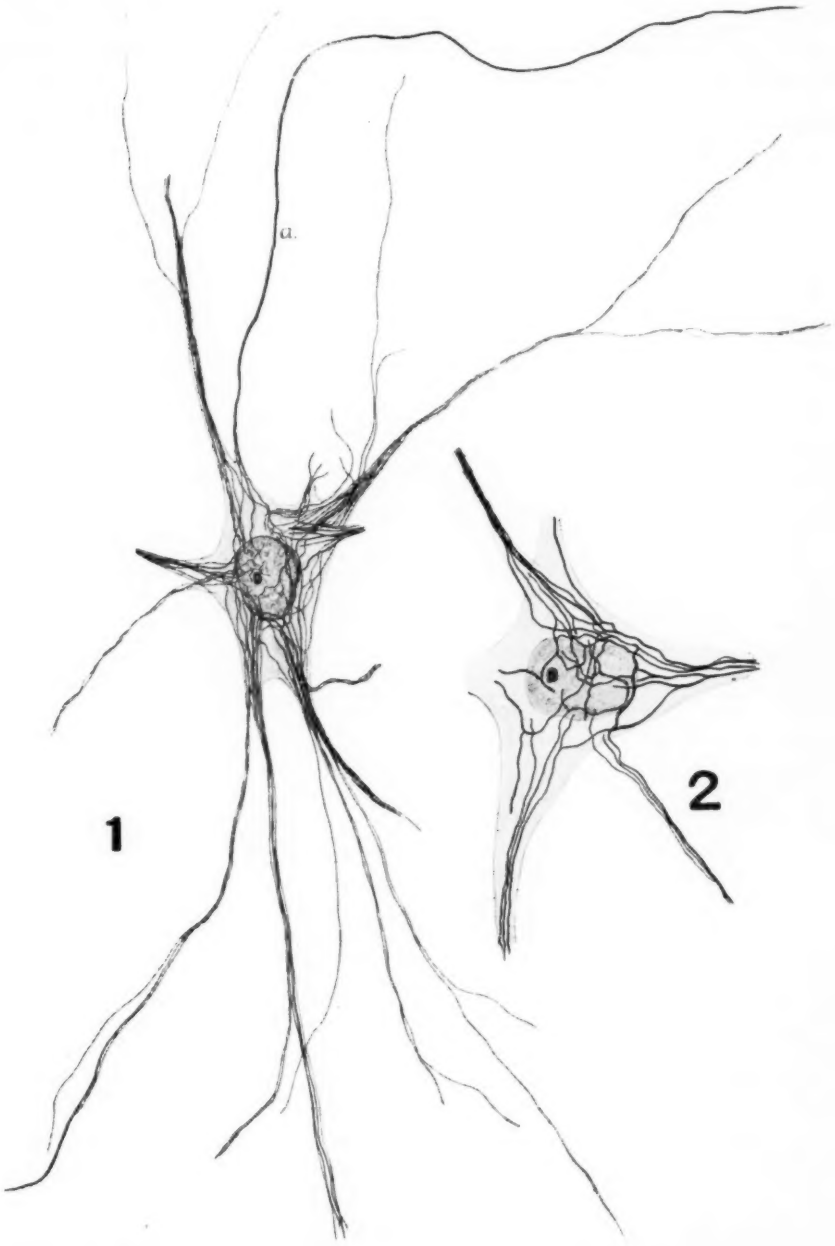
REFERENCE TO PLATE.

Figure 1. Motor cell from anterior horn of spinal cord (guinea-pig). The dendrites are relatively short structures; into these dendrites run fine fibrils (collaterals from other neurones), and these, retaining their individuality, become identical with the neurofibrillae of the cell. The axon (a) and fibrillae, when traced outwards from the cell, all end sooner or later, but such ending is due to the fact that they emerge from the plane of the section. If this cell had been stained by the Golgi method, it would have stained a uniform black, and the individuality of the collaterals, once they had entered the cell, could no longer have been recognized.

(Cajal's reduced silver method.)

Figure 2. Nerve cell from spinal cord (young rabbit). The focus has been made some distance above the nucleus. The anastomosing network around the nerve cell is evident. Neurofibril discontinuity within the cell is the result of cutting through of the fibrillae.

(Cajal's reduced silver method.)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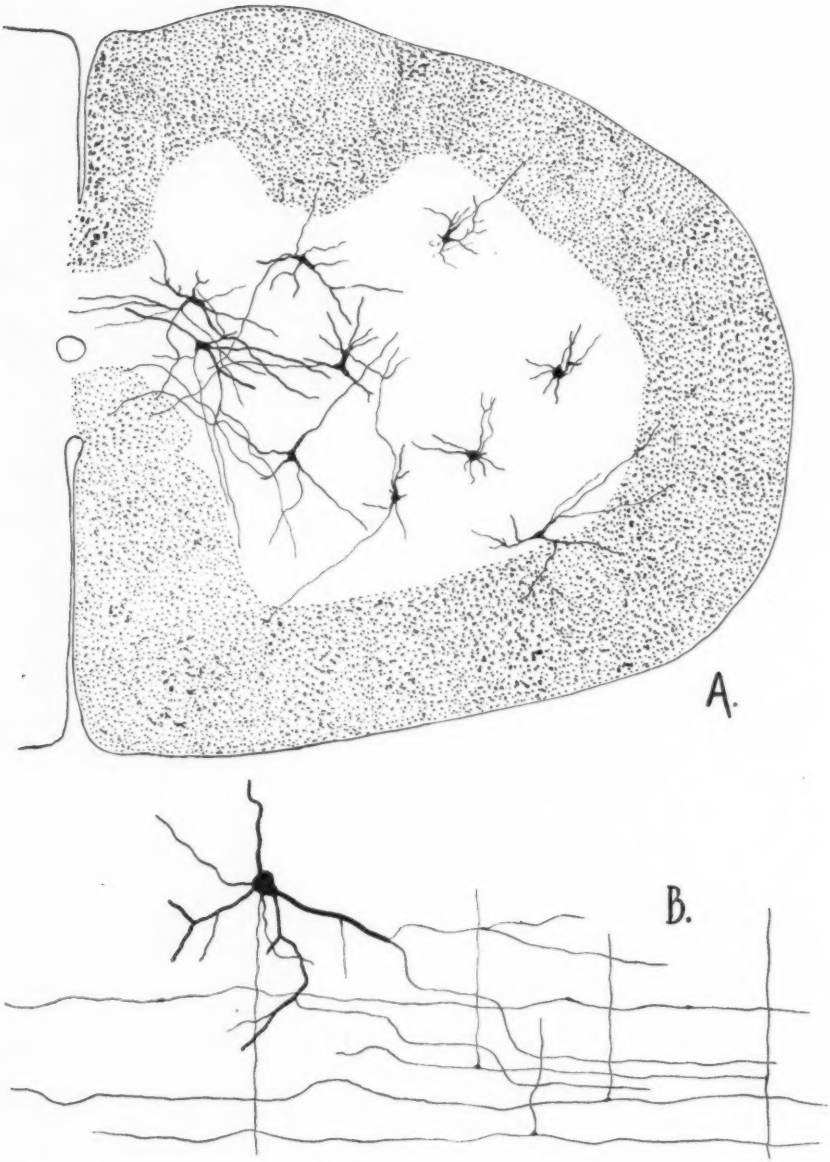
the relation of neurone endings to the following neurone of the reflex chain; for what has been called a dendrite is for its greater length merely a group of neurofibrillae that are approaching the body of the nerve cell in a bundle, and are entering it by way of its dendrites. Since the various technical procedures seem at first sight to yield such diverse results, it will be best to discuss this question first.

If we examine preparations made by Bielchowsky's method, or preparations made by Cajal's process (but not too strongly stained), then, as pointed out above, we can observe neurofibrils converging from various regions of the grey matter, entering the dendrites singly or in bundles, and then, retaining their individuality, approach the middle of the nerve cell (see figure 1). The neurofibrils may be followed, often for incredible distances, outwards from the cells upon which they have converged; eventually, however, they end suddenly, *but such ending is invariably due to the fact that they pass out of the plane of section.* In other words, it is not possible to demonstrate any neurofibril discontinuity in the spinal cord by the reduced silver method; the fibrils merely end because they have been cut through in making the section.

If we now examine the anterior horn cells stained by Golgi's process, an apparently different picture is revealed; the cells appear a homogeneous black, and neurofibrillae are not visible within them. From the "body" of the cells enormously long processes grow out, and these processes in their turn give off long, though very fine, secondary processes (see figure 9). If the sections are thick we can follow the cell processes for very great distances; nevertheless, they all end eventually, but almost always, *because they have been cut short.* (The few cases in which a true termination of the fibrils is observed might readily be due to incomplete staining.) With methylene blue preparations the same can be seen. The numerous figures given in Cajal's book are too well known to need further description. But in describing these anterior horn cells as cells with extremely long dendrites, the fundamental fact is constantly overlooked that the termination of the dendrites is not a real termination, but is merely due to the fact that they must inevitably be cut short as they eventually emerge from the plane of section of the preparation.

The Golgi preparations are accordingly to be interpreted in the following manner: Collaterals, consisting of one or more (?) neurofibrils, enter the grey matter from the white substance of the cord, and make their way towards the anterior horn cells. As they approach nearer the anterior horn cells, delicate fibrils join them, and the bundle grows in thickness; eventually it merges into the relatively short outgrowth from the nerve cell, and this short outgrowth constitutes the real dendrite of the cell. The reduced silver methods reveal the real nature of these neurofibril paths; the Golgi process, on the contrary, failing as it does to render visible any of the inner details of the "dendrites," presents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to us merely the picture of a heavily stained, highly branched cell, and is incapable of showing that the fine fibrils that join the collaterals as they approach the anterior horn cells retain their individuality right through the dendrites into the middle of the cell.

In further criticism of the synapse theory as applied to the *anterior horn cells*, it may be pointed out, firstly, that no convincing case of nerves terminating on the *body* of the nerve cells has ever been depicted. (Cajal's book gives a figure from a motor cell of the medulla, showing numerous minute knobs terminating on the nerve cell. See figure 112 of his book. The illustration is unconvincing, especially in the face of so much evidence leading to the opposite conclusion. The knobs might readily be regarded as endings of neuroglia fibres, for Cajal does not show them to be continuous with nerve fibres. But an even more probable explanation is that they are coagulations, rendered apparent as the nerve cell shrank during fixation. I have seen similar structures, especially with the use of the methylene blue process, and these knobs were certainly not connected with nerve fibres.) Numerous authors, in consequence, are inclined to figure synapses diagrammatically as brush-like terminations of neurones, ramifying among the *dendrites* of nerve cells, rather than terminating in close contact with the body of the cell. For example, the diagrammatic representation of a synapse, as originally presented by Michael Foster (4, figure 97), is partly conceived in this manner. But we should remember that the "dendrites" of a single nerve cell, if traced to their "termination," may ramify over a region occupying sometimes nearly half the area of the complete section of the cord (see, for example, figures 221 and 223 of Cajal's book; the figures refer to the medulla oblongata), and, to be consistent, the brush-like terminations should be of similar dimensions. But it is quite certain that a synapse of this size has never been observed.

It has been pointed out above that in reduced silver preparations the bundle of neurofibrillae that enters a dendrite of a nerve cell may stain a deep black, no internal differentiation into its constituent neurofibrils being visible. In such preparations the neurofibrils that compose the bundle are not visible till

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Figure 9. Sections of spinal cord (Golgi method).

A. Transverse section of spinal cord at its upper cervical end (cat). The figure represents a typical Golgi preparation, the nerve cells staining deep black, and possessing many long, at times branching, processes frequently called "dendrites." If the nerve cells of this preparation are compared with the nerve cell shown in figure 1, it will be noted that the "dendrites" of the Golgi preparation are composed of two elements, namely the true dendrites (which are relatively short) and the nerve fibrils (which enter the dendrites); but the Golgi method is not usually capable of rendering the distinction evident. In all cases the "dendrites," when traced outwards, terminate merely because they pass out of the plane of the section.

B. Longitudinal section of part of spinal cord; posterior horn (rabbit). In this preparation the distinction between the dendrite and nerve fibril seems fairly evident, although, of course, even here the fibrils cannot be followed into the cell. Four longitudinal fibres from the white matter are also shown.

the bundle has penetrated the dendrite, when they spread out in the form of a brush. If such a nerve cell were cut in section in such a way that the brush-like expansion of the bundle of neurofibrillae were cut through, the effect would be a structure having a very close resemblance to a branching discontinuous synapse. It is possible that preparations of this kind have lent support to the synapse theory.

We must conclude, then, that the reduced silver methods reveal a true neurone continuity on the anterior horn cells, and the results obtained by the Golgi and Ehrlich methods are not at variance with this conclusion.

Cells of the Posterior Horn and Clark's Column.

When the nerve cells of the posterior horn or of Clark's column are examined, stained by either of the reduced silver methods, precisely the same type of neurone junctions may be observed here, as on the anterior horn cells (see figure 3); if moderately thick sections are examined the fine fibrils may be traced into the cells, sometimes from very long distances. But again we notice, as with the anterior horn cells, that when we trace such fibrils outwards from the cells, they terminate only when they pass out of the plane of the section; again, therefore, no neurone discontinuity can be demonstrated on any of the Clark column or posterior horn cells.

In many preparations it may be observed that the neurones which terminate upon these cells are considerably thicker than a single neurofibril; nevertheless, no fine neurofibrillae can be seen joining them, to which their thickness might have been ascribed. We are therefore not yet able to state whether these fibrils that merge into and are continuous with the nerve cells of the cord are composed always of neurofibrillae that have converged from *various regions of the cord*, or whether they may at times constitute a small bundle of neurofibrils that have all grown out from a single nerve cell.

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Figure 3. Nerve cell from Clark's column (young rabbit). Note the various nerve fibrils entering the dendrites; the anastomosing network is not recognizable in this preparation. The outer termination of all the fibrils is due always to their leaving the plane of the section of the preparation.

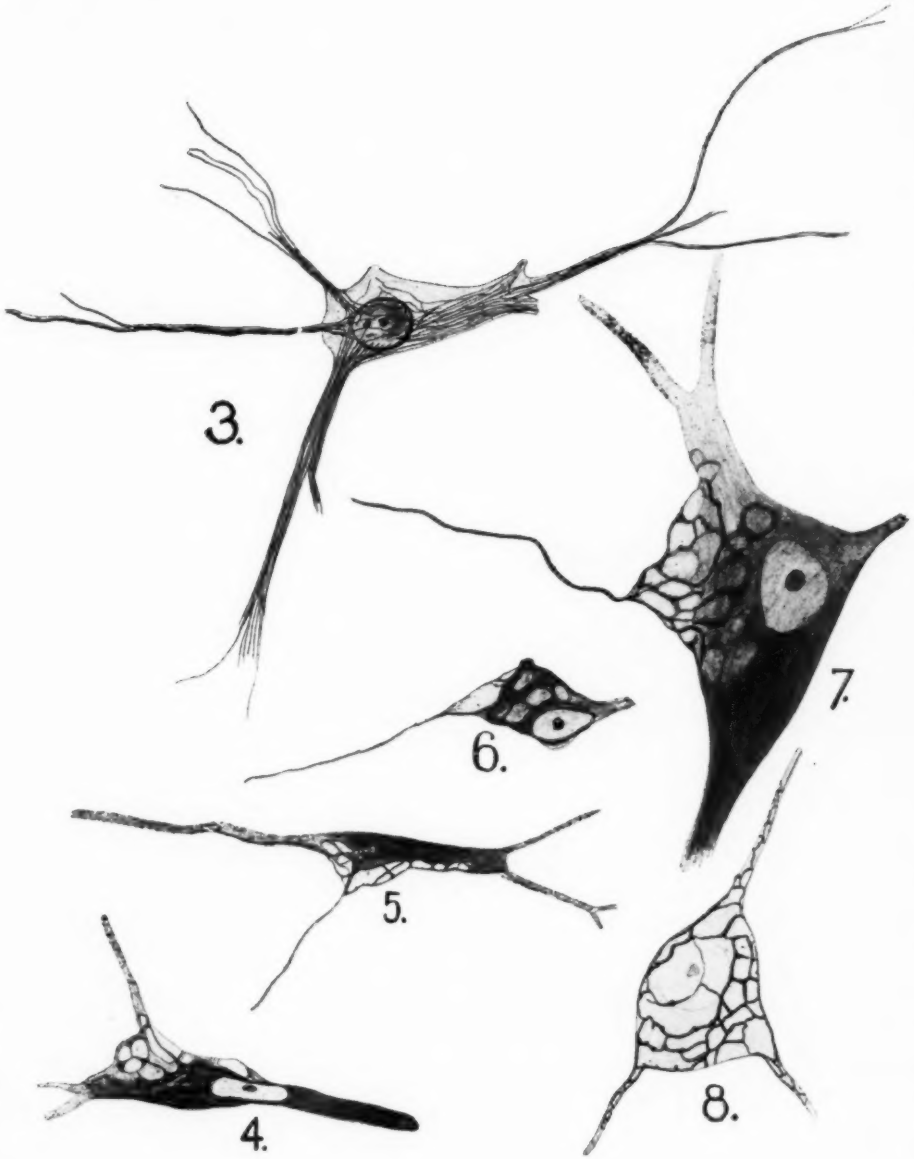
(Cajal's reduced silver method.)

Figures 4, 5, 6, 7. Four nerve cells from spinal cord of frog (*Hyla aurea*). In figures 4 and 6 the conical portion of the cell, which represents the region of outgrowth of a dendrite, has become vacuolated. In figure 5 is shown such a nerve cell, in which the vacuolated dendrite has assumed somewhat the appearance of a ramifying synapse. In figure 7 is shown such a nerve cell, with a highly vacuolated dendrite, giving the appearance of a neurone termination by a ramifying ending, upon the body of a nerve cell.

(Ehrlich's methylene blue method.)

Figure 8. Nerve cell from posterior horn (frog), to show the "superficial Golgi network," an obvious artefact due to coagulation.

(Ehrlich's methylene blue method.)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Effects Produced by the Metheylene Blue Method.

The main criticism that may be directed against the methylene blue process is that the tissues are never subjected to any process of fixation. We find, consequently, in preparations made by this method that the tissues present numerous obvious artefacts. The gelatinous material occupying so much of the region of the cord between the nerve cells may often present a high degree of vacuolation, and on some of the nerve cells a similar vacuolation may at times be seen. Sometimes, however, the cells stain remarkably clearly, despite the absence of fixation. Now it may be observed in such vacuolated preparations that a single dendrite may contain within it a single large vacuole, or several smaller vacuoles may be present, or a considerable number of very small vacuoles, which give the dendrite a frothy appearance, and such a dendrite may present an extraordinary resemblance to the branching dendrites figured by Cajal as occurring on the cells of Clark's column. It is possible that Cajal's branching dendrites are to be conceived in this light. In figures 4, 5, 6, and 7 a small series of dendrites stained by the methylene blue process are shown, and a gradation from an obviously vacuolated dendrite to one having a close resemblance to the "synapse" of Cajal may readily be observed.

In other cases I find that the surface of the cells presents very obvious coagulation artefacts, and modifications of such surface coagulations may show a marked resemblance to the structures figured by Cajal, Bethe, and others, and called by them the *superficial Golgi network* (figure 8).

The objections that are usually raised against the theory of neurone continuity are, firstly, that transsection of a neurone in a living animal causes degeneration of that neurone along all that portion of the fibre distal to the nucleated part of the cell, the degeneration extending up to but not beyond the next neurone of the reflex chain; secondly, that a reflex arc can conduct in only one direction.

The first objection is a weighty though not an insuperable one. The second does not carry much force, for, even assuming the existence of neurone discontinuity, we are unable to say why such discontinuity should limit the passage of impulses always to the forward direction, unless, of course, we assume the existence of a semipermeable membrane at the termination of such a discontinuous synapse, an arrangement which might equally well be found in a continuous conducting system—continuous, at any rate, so far as the microscope can reveal.

At the same time it seems fairly clear that the processes occurring within the "nerve cells" differ in some respects from those that occur in the fibres. Mosso concluded many years ago that the temperature of nerve cells rises markedly when they function actively, and the rapid fatigue that ensues in nerve cells deprived of oxygen is in marked contrast to the relative absence of fatigue in nerve fibres placed under similar conditions.

From these considerations it seems that we must conclude that the processes which occur within the nerve "cell" during excitation, are not entirely similar to those which occur within the nerve trunks, and when such special properties of the nerve cell can be demonstrated, it would seem that the property of forward conduction of the reflex arc could be attributed to these, and that a visible discontinuity in the conducting system need not be postulated.

SUMMARY.

1. By means of the reduced silver methods of Bielchowsky and of Cajal, it is found that the fine collaterals that enter the grey matter from the white matter of the spinal cord converge upon the nerve cells of the grey matter, and penetrate the dendrites of the nerve cells, being continuous with, and indeed quite indistinguishable from, the neurofibrils of the nerve cell. These fine collaterals, having entered the nerve cells, pass independently of one another, as the neurofibrils, towards the middle of the nerve cell, where they anastomose. From this anastomosing system a small number of neurofibrils arise, and emerge through the axon.

2. The anastomosing system appears to be the seat of the integration, with the component phenomena of summation, inhibition, fatigue, delay, one-directional conduction, etc.

3. It is shown that preparations made by the methods of Golgi and of Ehrlich lead to similar results, much obscured by the fact, however, that they do not usually render visible the internal differentiation of the nerve cells and dendrites. Dendrites are relatively short structures, and the greater portion of the enormous "dendrites" made apparent by the Golgi method are in reality bundles of neurofibrillae, whose real nature is obscured through a defect in the method of staining.

4. Ramifying synapses, coming into close contiguity, but not into direct continuity with the nerve cells, do not seem to occur in the spinal cord. It is suggested that the ramifying synapses of Cajal, and the superficial Golgi network of Bethe, are artefacts due to coagulation and frothing of the protoplasm.

5. It is concluded, therefore, that a true neurone continuity occurs at the junction of neurones in the spinal cord, but such neurone continuity is not a dendrite continuity of the type originally described by Gerlach, nor a continuity through neurone collaterals of the type upheld by Golgi and Nansen, nor yet a continuity through a superficial Golgi network, as maintained by Bethe, but a continuity in which collaterals from various neurones penetrate the various dendrites, and, passing into the substance of the nerve cells, anastomose, the anastomosis with collaterals from other neurones thus permitting evidently of integration, and the whole of the integration occurring within the body of the nerve cell.

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STUDIES ON THE REFRIGERATION OF MEAT

II. THE FREEZING OF BEEF AND MUTTON PRESS JUICES.

by

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(Submitted for publication 3rd May, 1926.)

1. INTRODUCTION.

In a previous paper (2) experiments on various factors affecting the degree of reversibility of beef during the freezing and thawing cycle were described. In these experiments one of the most important factors was found to be the rate of freezing, but complete reversibility, as measured by the amount of drip exuding from a cut surface, was obtained only when freezing was almost instantaneous, liquid air being used as the freezing medium. In all other experiments, when the latent period of freezing was reduced to as low as 0·4 hour, there was still an appreciable quantity of drip on thawing. In the refrigeration of animal muscle changes may be produced both in the contents of the muscle fibre (muscle plasma) and in the surrounding membrane (the sarcolemma), and alterations in the latter may bring about changes in its permeability. The presence of drip in beef after freezing and thawing may therefore be due to irreversible changes either in the plasma or in the sarcolemma, or in both. With the hope of throwing further light on the problem, experiments have been carried out with the juice expressed from minced muscle. Although the colloidal system within the fibre is non-homogeneous, the juice obtained in the above manner approximates to the muscle fibre contents.

Press-juice from ox muscle was used in most of these experiments, but comparative tests were also made with mutton press-juice, to ascertain if the well-known differences in the properties of beef and mutton during freezing and subsequent thawing, are due to differences in the behaviour of the muscle plasma.

2. EXPERIMENTAL METHODS.

(a) Preparation of the Press-juice.

Attempts were made to prepare a sample of juice from the muscle before rigor mortis had set in, as it was thought that a more representative sample could be obtained in this way. Owing to the fact that the whole muscle was in a

jelly-like state immediately after death, it was found impossible to express any appreciable quantity of juice, 60 cc. being the maximum amount obtained from 6 kgm. of meat. In all experiments, therefore, juice was obtained about 48 hours after rigor mortis had passed off.

The muscle was freed from fat and connective tissue, finely minced, and ground with clean, fine sand. The pasty mass was then pressed in a filter press, and a viscous, clear, red liquid was obtained. This liquid was centrifuged for twenty minutes at about 2,000 revolutions per minute. The total nitrogen content of the supernatant liquid was determined in 5 cc. by Kjeldahl's method. This was found to vary in the several series from 1·30 to 1·80 gm. per 100 cc. of juice. A few drops of toluene were added to prevent bacterial action.

(b) Freezing.

As soon as possible after centrifuging, definite volumes of the juice were frozen in uniform cylindrical glass vessels, using as the freezing media either air, a saturated solution of calcium chloride, or mercury, at temperatures which were arranged to give the desired rate of freezing. Different rates of freezing were obtained also by using cylindrical vessels of different diameters in the same medium. Each vessel was fitted with a thermo-junction (either glass-covered or bare), held in position in the centre of the juice by corks, as shown in figure 1. This gave the minimum rate of freezing for the sample of juice, and the cork stoppers prevented evaporation during storage in the frozen condition. Supercooling was obviated by gently shaking the contents of each vessel every few minutes.

(c) Thawing.

Thawing of the frozen juice was carried out either in air or in water, so that the rate could be regulated to give very fast or very slow thawing. When thawing was completed, 1 cc. of each sample was removed for ultramicroscopic examination. The juice was immediately centrifuged under the same conditions as were previously used. This process precipitated all the small "dehydrated" masses which were suspended in the juice. Since most of the colloidal material consisted of protein and their derivatives, it was safe to assume that the precipitated material was mainly nitrogenous in character. The total nitrogen of the supernatant liquid was then estimated in 5 cc. as before. The greater the degree of reversibility, the greater will be the total nitrogen content (i.e., the total soluble protein) of this supernatant liquid, complete reversibility being shown when the nitrogen content of the supernatant liquid is equal to the content before freezing. The results have been expressed as the ratio of the total nitrogen content of the supernatant liquid after freezing and thawing to the total nitrogen content of the fresh juice.

(d) Examination with the Ultra-microscope.

It was suggested that if actual ultramicroscopic examination of the juice were carried out, both before freezing and after thawing, an indication might be obtained as to the extent of the coagulation, if any, taking place during the

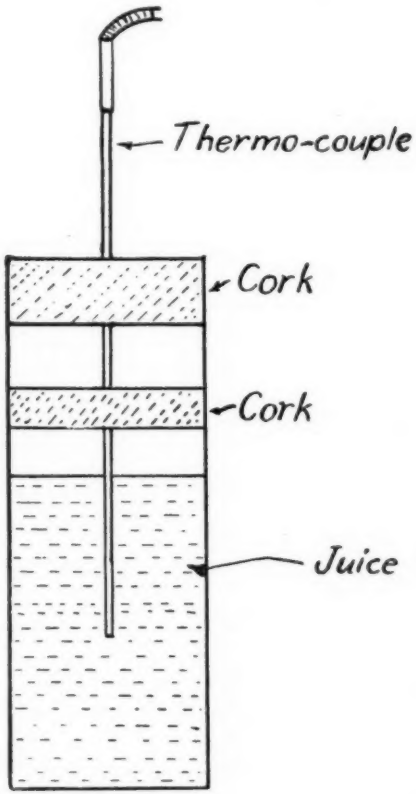

Figure 1.

freezing and thawing cycle. For these observations the ultramicroscopic apparatus of Zeiss was used in conjunction with a cardioid condenser. Since the numbers of particles observed in a given field will vary with the degree of illumination, after setting up the apparatus and adjusting the illumination to a convenient strength, it was not disturbed until all the experiments were completed. It was assumed that slow freezing would cause an aggregation of

the single particles, and the slower the freezing the less would be the number of single particles remaining in the fluid. Hence, a comparison was made by counting the actual numbers of single colloidal particles possessing marked Brownian movement, contained in a given volume before and after freezing and thawing. This method was open to criticism, since, in some cases, freezing may cause an actual disappearance of large numbers of single particles which may form aggregates containing two or three. These aggregates, still possessing strong Brownian movement, would not separate out when the juice was centrifuged. Hence, although there would be a distinct diminution in the numbers of single particles remaining, the degree of reversibility, as measured by the first method, would be great. Unfortunately, no more satisfactory method of estimating the degree of reversibility could be found. The ultramicroscopic examination was carried out in the case of one series only.

3. RESULTS.

(a) Changes in Nitrogen Content.

In the frozen condition, the samples of juice which had been slowly frozen in air showed numbers of fairly clear, wide ice crystals stretching from the sides of the cylinders. On the other hand, the samples which had been frozen more quickly, for example, in calcium chloride, showed numerous, very small crystals scattered throughout the solid mass. After thawing had been completed the more slowly frozen samples had extensive brownish-coloured precipitates of "dehydrated" protein, with small particles suspended in the liquid, whilst the faster frozen juices had small precipitates. In both cases these precipitates appeared immediately after the frozen system had begun to liquify. Stiles (1) states that this flocculation is exhibited by many colloidal solutions which have been frozen and thawed, such solutions showing numerous flocks, which gradually precipitate after thawing.

TABLE 1.

Expt.	Diam. of Cylinder. cm.	Freezing Medium.	Freezing Temp. Degrees C.	Freezing Latent Period. Hours.	Thawing. Medium.	Thawing. Temp. Degrees C.	Thawing. Latent Period Hours.	Ratio. $\frac{a_1}{a_2}$
1	3.5	Air	—12.5	3.5	Water	20	0.25	0.76
Beef Juice	3.5	Air	—12.5	3.5	Air	21.5	1.1	0.84
75 cc.	3.5	$CaCl_2Aq.$	—12	0.5	Water	20	0.25	0.95
Samples	3.5	$CaCl_2Aq.$	—12	0.5	Air	21.5	1.1	0.96
2	3.5	Air	—14	3.0	Air	13	2.1	0.95
Beef Juice	3.5	Air	—14	3.0	Air	1	9.5	0.95
50 cc.	3.5	Mercury	—4	1.1	Air	13	2.1	0.96
Samples	3.5	Mercury	—4	1.1	Air	1	9.5	0.95
	3.5	$CaCl_2Aq.$	—14	0.3	Air	13	2.1	0.96
	3.5	$CaCl_2Aq.$	—14	0.3	Air	1	9.5	0.95

Expt.	Diam. of Cylinder. cm.	Freezing. Medium.	Freezing. Temp. Degrees C.	Freezing. Latent Period. Hours.	Thawing. Medium.	Thawing. Temp. Degrees C.	Thawing. Latent Period Hours.	Ratio. $\frac{a_1}{a_2}$
3	3·5	Air	—10	3·0	Air	13	2·1	0·95
Mutton	3·5	Air	—10	3·0	Air	1	9·5	0·94
Juice	3·5	Mercury	—4	1·1	Air	13	2·1	0·96
50 cc.	3·5	$CaCl_2Aq.$	—14	0·3	Air	13	2·1	0·96
4	7·0	Air	—12	5·4	Water	7·5	0·5	0·84
Beef Juice	7·0	Air	—12	5·4	Air	4	—	0·90
50 cc.	3·5	Air	—12	3·9	Water	7·5	0·35	0·88
	3·5	Air	—12	3·9	Air	4	—	0·92
	3·5	$CaCl_2Aq.$	—13·8	0·35	Water	7·5	0·35	0·92

The results of this method of testing reversibility are given in Table 1, which gives the conditions of freezing and thawing to which the juices have been subjected. The last column gives the ratio of the percentage of nitrogen in the thawed supernatant liquid (a_1) to the percentage in the fresh juice (a_2).

These results showed that the maximum reconstitution of the beef juice attainable in these experiments was 96%, which value was observed with latent periods of freezing up to three hours. For these latent periods the reconstitution is not affected by the rate of thawing. On the other hand, in the experiments where this latent period was exceeded (experiments 1 and 4), reconstitution was aided by slower thawing. In experiments 2 and 3, in which comparisons were made between the juices from beef and mutton, for latent periods of freezing up to three hours, no difference was observed, both yielding the greatest degree of reversibility which it was apparently possible to attain.

(b) Examination with the Ultramicroscope.

This procedure was used in the cases of experiments 2 and 3, Table 1. An eye-piece was employed with a field divided into squares, and the numbers of single colloidal particles in one square were counted. Since the same depth (2μ) of fluid was always used, the results were strictly comparable. The results were expressed (in Table 2) as the ratio $\frac{A_2}{A_1}$ of the number of single particles in a given volume of thawed juice (A_2) to the number in the same volume of fresh juice (A_1). Fifty counts were made in each experiment, and the average values recorded. The samples of fresh beef and mutton juices contained an average of 6·65 and 11·70 single particles respectively in the given volume examined. These figures refer to the term A_1 in the ratio.

TABLE 2.

Juice.	Latent Periods. Freezing. Hours.	Thawing. Hours.	A_2. Number of single particles in vol. x of thawed juice.	Ratio. $\frac{a_1}{a_2}$
Thawed Beef Juice	3.0	2.1	3.2	0.48
" " "	3.0	9.5	3.8	0.57
" " "	1.1	2.1	3.8	0.57
" " "	1.1	9.5	5.1	0.76
" " "	0.3	2.1	4.3	0.64
" " "	0.3	9.5	5.6	0.84
Thawed Mutton Juice ..	3.0	2.1	10.7	0.90
" " "	3.0	9.5	11.0	0.93
" " "	1.1	2.1	10.4	0.88
" " "	0.3	2.1	11.0	0.93

The slower freezing of beef juice caused quite a considerable number of single particles to disappear. The smaller number of particles probably indicated the formation of aggregates too small to be precipitated by centrifuging, since the figures for the nitrogen contents quoted in Table 1 showed that almost perfect reconstitution had been attained in all the experiments having a latent period of 3 hours and under. Even in the beef juice having a latent period of freezing of 0·3 hours, considerable numbers had disappeared. Slow thawing was found to have allowed time for a greater redispersion of particles.

In contrast to beef juice, mutton juice showed very little disappearance of particles, even with latent periods of freezing up to 3 hours. This result may be attributed to greater difficulty in redispersing the beef colloid aggregates formed on freezing, owing possibly to a greater degree of "dehydration" taking place in the colloidal particles of beef juice.

4. DISCUSSION.

In a review of the process of the freezing of tissues, Plank (4) stressed the importance of two effects of freezing, viz., the damage to the protoplasmic structure by the formation of ice crystals, and the consequent "dehydration" of colloid which is entailed. He also pointed out the importance of what he considered to be the two main factors in determining whether reconstitution of the protoplasm would take place, viz., the rate of freezing and the temperature to which the frozen substance is cooled. If the results of the experiments on the freezing and thawing of beef juice can be applied to the muscle plasma, they tend to show that Plank has omitted one very important factor in his theory of the freezing of muscle tissue, viz., the properties of the sarcolemma during freezing and thawing. Practically complete reversibility on thawing may be obtained by freezing beef juice at such a rate as to give a latent period of 3·0 hours; with the tissue as a whole (plasma and sarcolemma) the same degree of reversibility is not attained until the latent period of freezing has been reduced to at least 0·5 hours. Hence, it would seem as if the sarcolemma plays a very important

rôle in determining the amount of reconstitution which is to take place. Possibly an increased permeability of the sarcolemma takes place on exposure to temperatures of 0°C. and below, for Foster and Moyle (3) state that such increased permeability would account for the loss of irritability which takes place when frogs' muscles are exposed to a temperature of 0°C. for a long period. The slight differences between beef press-juice and mutton press-juice on freezing at a speed which would give a latent period of 3·0 hours, seem insufficient to account for the differences in the properties of beef and mutton during freezing, and suggest that the difference lies to a considerable extent in the changes of the sarcolemma.

SUMMARY.

1. The determination of the amounts of unprecipitated nitrogen before freezing and after thawing, showed that fairly complete reconstitution of beef juice was attained with rates of freezing which gave latent periods of 3 hours and under.

2. This reconstitution was aided by slow thawing.

3. Very little difference between beef and mutton juices could be detected by this nitrogen content method.

4. Ultramicroscopic examination of beef juice showed that the freezing and thawing cycle had caused large reduction in the numbers of single colloidal particles. The higher the rate of freezing, however, the less was the reduction.

5. Mutton press-juice, unlike beef juice, showed very little diminution in the numbers of single colloidal particles, but the variation of the rates of freezing from 0·3 hour to 3 hours did not produce any material alteration in the numbers.

6. The results of the two methods may be reconciled by supposing that during the freezing process the colloidal particles of beef juice may have formed small aggregates, which would not be precipitated by centrifuging.

7. The results suggest that changes which take place during the freezing of beef are largely determined by alterations in the sarcolemma of the muscle during freezing.

8. The differences between the properties of beef and mutton during freezing and thawing may be partly due to differences in the properties of their respective sarcolemma.

In conclusion, I desire to thank Associate Professor W. J. Young for his many helpful suggestions and constant interest in the investigation, and Mr. G. A. Cook, of the Commonwealth Institute of Science and Industry, for performing many of the physical measurements.

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STUDIES ON THE MITOCHONDRIA OF PARAMOECIUM

by

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(Submitted for publication 7th May, 1926.)

The investigation reported in this paper was directed towards determining the behaviour of the mitochondria of Paramoecium during the life cycle of the organism, with a view to helping to elucidate the question of the nature of these bodies and the part they seem to play in the life of the cell. The results obtained in this research seem to show (a) that the mitochondria are true cytoplasmic bodies, and not merely coagulations of the cell protoplasm following upon fixation; (b) that no evidence is yet available to show that they are formed *de novo* from the cytoplasm, but that on the contrary they always increase in number by binary fission; (c) that the occurrence of fission in the mitochondria is irregular, and is quite independent of the occurrence of fission or conjugation in the organism; (d) that they are concerned, on the contrary, with cell metabolism, playing an important part in the process of intracellular digestion (this latter observation will serve to correlate our knowledge of the function of the mitochondria in a unicellular organism, and in the cells of higher animals, where they give rise to extracellular enzymes); (e) taking into account the theory that they are enzymatic in nature, and my previous observations on *Opalina*, where they appear to be the centres of protoplasmic synthesis within the cell, their behaviour appears to illustrate the capacity of enzymes to reverse their activity in accordance with conditions, i.e., synthesis or hydrolysis, according to concentration of the substrate.

Methods of Investigation.

For rendering visible the mitochondria of Paramoecium, the janus green vital stain may be employed; or organisms, fixed in osmo-chromic fluid, may be cut in section and stained with iron haematoxylin.

The former method is particularly useful in dealing with cases in which only a single individual of a thick culture is in the particular stage of the life cycle of the organism that is at the time required for investigation, such, for

example, as the conjugating phase, which occurs only very rarely. It is practically impossible to treat such isolated individuals by the haematoxylin method.

The second method yields much more illuminating cytological results. A pure culture of Paramoecia, grown in a solution of malted milk (1 gram per litre), is fixed with the osmo-chromic mixture, or in Flemming solution (omitting the acetic acid). After fixing, the culture is centrifuged and the fluid poured off. By this means the Paramoecia are passed through alcohol, cleared, and eventually embedded *en masse* in paraffin, and then sections cut, which are subsequently stained with Haidenhein's process. When very large numbers of individuals, prepared by this method, are examined, organisms in the various stages of the life cycle are readily recognized, and their mitochondria may be studied at leisure. By this means I have examined the mitochondria during all stages of the life cycle, except the conjugation phase, which I have only studied in living specimens stained with Janus green.

The greatest source of error in mitochondrial work is that it is usually very difficult to distinguish mitochondria from rod-shaped bacteria. But a ready test is available: mitochondria fixed for 24 hours in absolute alcohol are not capable of subsequent staining with iron haematoxylin; bacteria, on the contrary, are not visibly affected by this treatment with alcohol. The mitochondria do not entirely dissolve when placed in alcohol, for they can often be clearly seen in sections made from such material, but they are incapable of being stained by haematoxylin.

DESCRIPTION OF PLATE.

All figures (except fig. 9) are drawn from osmo-chromic material, stained with iron haematoxylin.

Fig. 1. Longitudinal section of adult Paramoecium, showing organization of the mitochondria. Mitochondria near the surface of the organism are shown dark; those in the deeper part of the organism paler. Note the arrangement of the superficial mitochondria in longitudinal rows. Meganucleus and micronucleus clearly visible.

Fig. 2. Transverse section of an adult Paramoecium, containing six food vacuoles. Note the large food globules within the vacuoles. Note also the surface arrangement of the mitochondria; the mitochondria in the deeper parts of the organism are seen entering the vacuoles. Some of the vacuoles contain numerous mitochondrial bodies (not bacteria).

Fig. 3. Longitudinal section of same, showing five foot vacuoles containing numerous mitochondria (not bacteria). Note also mega- and micro-nucleus.

Fig. 4. Paramoecium undergoing binary fission.

Fig. 5. Paramoecium, showing commencement of fission of micronucleus. The focus is along the middle of the organism.

In figs. 5 and 6 it is seen that although many of the mitochondria are in the dumb-bell shaped condition, the proportion of dividing to non-dividing mitochondria is not greater than usual; micronuclear division is not, therefore, accompanied by a general mitochondrial fission.

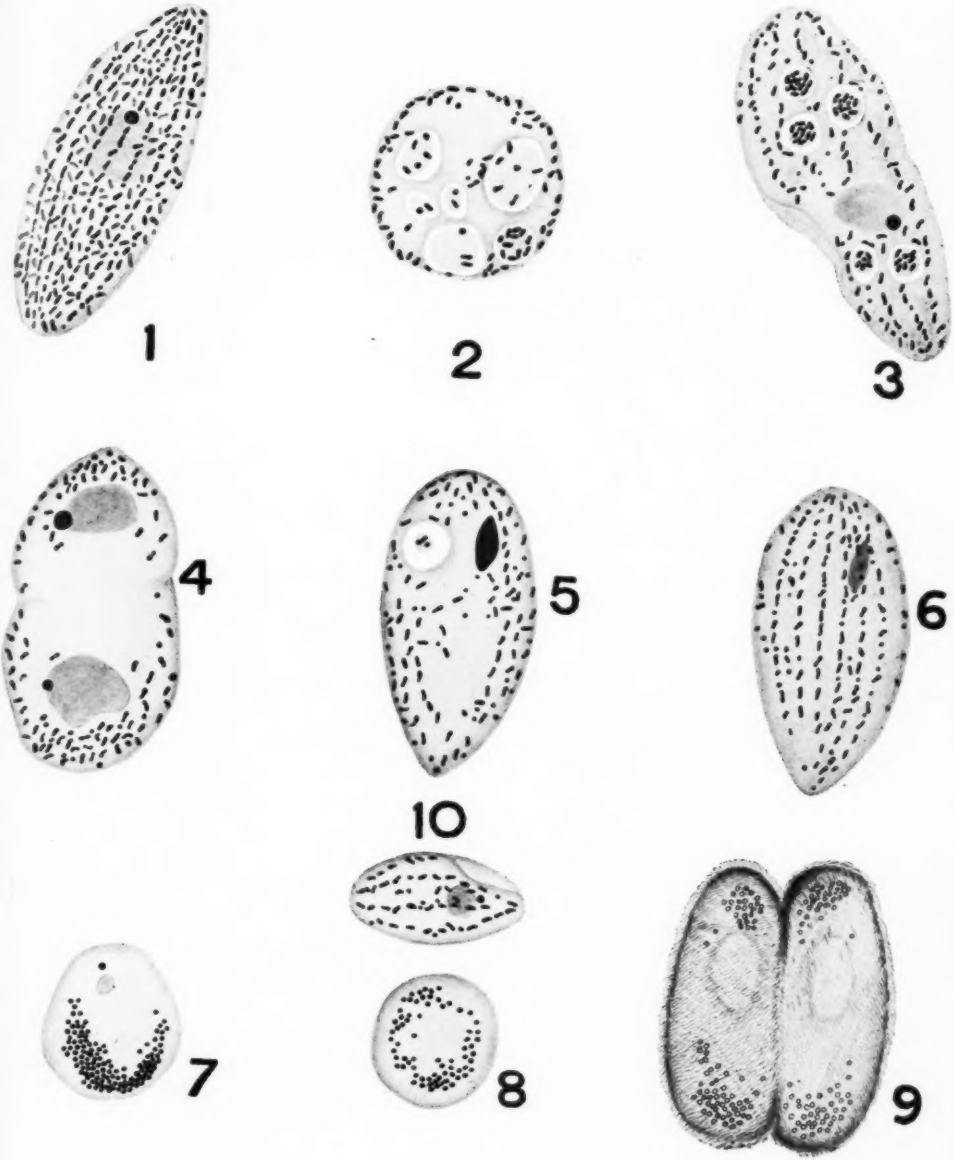
Fig. 6. The same; in surface view.

Fig. 7. An encysting Paramoecium, centrifuged for 1¾ hours before fixation. Note separation of mitochondria and nucleus.

Fig. 8. A normal cyst, not centrifuged.

Fig. 9. Two Paramoecia undergoing conjugation. Taken from two living individuals, stained with janus green (vital stain).

Fig. 10. Young Paramoecium, taken after fission.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The Mitochondria of the Adult Paramoecium.

When Paramoecia, cut in longitudinal section and stained with haematoxylin, are examined under high magnifications, the cytoplasm is found to contain a large number of rod-shaped bodies. If the organism is examined in its deeper parts, these rod-shaped bodies are seen to be disposed somewhat irregularly, and no distinct polarity is recognizable; but if the outer region of the organism is observed, it is found that these bodies are arranged one behind the other in rows, stretching right along the length of the organism (figure 1).

These bacteria-like bodies are the mitochondria. This statement is based on the following facts: (a) Similar bodies may be observed in the living organism when stained by Janus green; (b) Paramoecia fixed in absolute alcohol appear entirely devoid of these bodies; (c) when Paramoecia encyst naturally, or when encystment is induced in them by placing them in Ringer's fluid, the rod-shaped bodies as such disappear, and transform themselves into spherical bodies in precisely the same way as do the mitochondria of *Opalina* (5), and the mitochondria of the pancreas (4); (d) many of the mitochondria are undergoing, as will be described below, transverse binary fission of a kind similar to that observed in the mitochondria of *Opalina*. From these observations the conclusion is drawn that the rod-shaped bodies present in the cytoplasm in Paramoecium are identical with the bodies usually referred to as mitochondria.

Before describing the behaviour of the mitochondria during the life cycle it is necessary to draw special attention to the fact that we possess the very strongest evidence that these bodies are *genuine cytoplasmic inclusions, and not merely artefacts due to the action of the fixing fluid*. We have as evidence for this statement the following facts: (a) Highly refractive bodies, having a similar appearance to the mitochondria, may be observed, though not very distinctly, in living Paramoecia, if these are examined under high magnifications; (b) janus green is capable of staining these bodies in the living organism at a time when the cell protoplasm has not yet coagulated; (c) if a culture of living Paramoecia is strongly centrifuged for about $1\frac{3}{4}$ hours, the organisms gradually encyst; if such cysts, fixed in osmo-chromic, are now examined and compared with cysts that have been centrifuged only subsequent to fixation, it is found that in the former the mitochondria have largely separated from the rest of the cell protoplasm, and have accumulated at one end of the cyst, while the nucleus and micronucleus lie in a mass of clear, granule-free cytoplasm at the other (figure 7). It is obvious that in this case the granules can be separated from the rest of the cytoplasm, and when this cytoplasm is now coagulated with fixatives, it does not exhibit any granulation. A similar observation has been made by E. N. Browne (2) in the spermatocyte cell of *Notonecta*.

The Behaviour of the Mitochondria during the Life Cycle.

If very small individuals in a preparation made by the above method are examined—Paramoecia, that is, which have just been formed by the binary fission of the adult organism—it is found that the mitochondria have already the typical appearance as seen in the adult Paramoecium, i.e., they are rod-shaped bodies, longitudinally disposed in rows, on the more superficial parts of the protoplasm, but somewhat irregularly arranged in the deeper parts (figure 1). But we always observe in such organisms that the mitochondria are present in very small numbers.

As the Paramoecium grows, the number of mitochondria increases greatly, and such increase is evidently due to a binary transverse fission of the mitochondria, for many of the mitochondria may be observed in these growing organisms, in a dumb-bell shaped condition, in which they are evidently dividing into two parts. Eventually the adult condition shown in figure 1 is reached. There is no evidence to show that fission occurs simultaneously throughout all mitochondria in the Paramoecium; on the contrary, it occurs quite irregularly, and appears to be an inherent property of the mitochondria themselves, rather than the result of a force acting throughout the whole of the organism.

No evidence has yet been obtained to show that the mitochondria might possibly be formed *de novo* from the cytoplasm. It would, of course, be extremely difficult to exclude this possibility, but so far as I have been able to observe, they arise only by the fission of other mitochondria.

When the adult Paramoecium undergoes binary fission, the mitochondria merely pass, without undergoing fission, into the two daughter cells. Fauré-Fremiet has stated (3) that fission of the micronucleus is accompanied by a fission of the mitochondria, the rod-shaped bodies all undergoing fission. recognizable by their dumb-bell shape. But I find that in Paramoecium such a thing does not occur; many of the mitochondria are in the dumb-bell shape form, but the proportion of such dividing mitochondria is not greater than that found in the adult Paramoecium. The fact that the number of mitochondria in the small daughter cell is so very much less than that occurring in the adults prior to micronuclear fission is further evidence against the statements of Fauré-Fremiet.

The behaviour of the mitochondria in conjugating organisms, I have observed only in the living animal, stained alive with janus green. So far as I could observe, no interchange of mitochondria take place during conjugation, and the mitochondria do not seem to take any part whatsoever in the process (figure 9).

When the Paramoecium undergoes natural encystment, or when encystment is induced by placing them in Ringer's fluid, the mitochondria round themselves off, and are seen in the fixed cysts as numerous minute, strongly-staining, spherical

bodies, which apparently elongate again, when the organism once more assumes the free, swimming condition.

These observation seem to show, then, *that the behaviour of the mitochondria during the life cycle of the Paramoecium is in no way related to any genetic or sexual phenomena during the life cycle, so that it would rather suggest that the mitochondria were concerned with the general metabolism of the cell.*

The Function of Mitochondria in the Metabolism of the Cell.

There are a number of very significant observations in existence which seem to demonstrate that the mitochondria are bodies having an enzymatic nature. In 1909 Regaud and Mawas suggested that in the salivary glands the secretory granules arose from the mitochondria, thus implying their relationship to enzymes, while Arnold (1) observed the origin of the zymogen grains in the pancreas from mitochondria. Marston (6) has pointed ou that the behaviour of mitochondria towards azine dyestuffs is identical with the reactions which proteolytic enzymes undergo in the presence of these substances, and concludes that they may be the synthesizing enzymes within the cell, their function being to build up protein at a water-poor phase at their surface. In agreement with this I find that in the pancreas the zymogen grains, which evidently constitute the tryptic secretion, arise from the filamentous mitochondria, being simply constricted off from these (4), while in *Opalina* I find (5) that in close connection with each mitochondrium a large granule may develop, this granule being evidently the result of the local synthetic activity of the mitochondrium, formed as it is by the concentration of highly diffusible foodstuffs that have penetrated the cuticle of the organism.

In Paramoecium this apparent synthetic activity of the mitochondria cannot be detected with the microscope—possibly *Opalina* is a very favourable object for the investigation of this point. But in Paramoecium, feeding as it does in the true holozoic manner, a process of intracellular digestion, very similar to the process of extracellular digestion observed in gland cells, can apparently be readily demonstrated. If Paramoecium from an actively feeding culture, living in an extract of malted milk, are examined, the food vacuoles are usually seen to contain a large, rounded globule, this being evidently the coagulated milk from the culture fluid; but a very striking feature of such preparations is that these globules frequently contain a number of rod-shaped bodies, closely resembling bacteria, and also having the same appearance as the mitochondria (figure 3). They are not present in all the individuals of a culture; in one culture, for example, I found them in 76% of the individuals; in another culture only about 31%. The possibility that these bodies might be bacteria, engulfed in the food, or even symbiotic bacteria, seems excluded by the following two considerations: I find that they are present in the food vacuoles of Paramoecia reared in culture

fluid that was entirely devoid of bacteria; secondly, if such Paramoecia are killed in absolute alcohol, and then stained with iron haematoxylin subsequent to sectioning, these rod-shaped bodies within the vacuoles do not stain, though they are readily observed in controls prepared from the same culture and fixed in an osmo-chromic fixative. Thirdly, they are stainable by safranin, after osmo-chromic fixation, but alcohol fixation deprives them of their capacity for staining. These observations, and their obvious resemblance to the mitochondria of the cell protoplasm, are taken as strong evidence that these bodies are identical with the latter, a conclusion which is supported by the fact that some of them are in the dumb-bell shaped condition. In *Carachesium,* Fauré-Fremiet has observed that the mitochondria tend to cluster around the food vacuoles. Within the vacuoles these bodies seem gradually to disintegrate; some of them certainly have a fragmentary appearance. It is a matter of considerable difficulty to observe their entrance into the vacuoles; nevertheless, figure 2 seems to illustrate such a condition, some of the mitochondria entering, and others apparently approaching, the cell vacuole.

Assuming the conception of these bodies as constituting the cell enzymes to be correct, we seem here to have before us a phenomenon identical with that observed in pancreatic cells. In the pancreas digestion is extracellular, and the mitochondria are extruded as zymogen grains into the pancreatic tubules; in Paramoecium digestion is intracellular, and the mitochondria are extruded consequently into the food vacuoles. Since, moreover, there is no evidence that the enzymes are extensively used up during their activity, it would seem to follow that in Paramoecia, excretion of enzymes must occur from the Paramoecium into the surrounding culture fluid whenever the contents of the vacuoles are expelled to the exterior.

SUMMARY.

1. Mitochondria are not artificial products, due to the action of fixatives on the protoplasm, for (a) they may be seen in living cells, (b) they may be stained intra-vitam with Janus green, (c) they may be separated from the remainder of the cytoplasm by centrifuging.

2. During the growth of the Paramoecium, the rod-shaped mitochondria increase by undergoing transverse binary fission; they undergo such fission irregularly, and apparently independently of the occurrence of genetic phenomena during the life cycle. At cyst formation they round themselves off. There is no evidence available to show that they arise *de novo* from the cytoplasm.

3. Mitochondria are extruded from the cell protoplasm into the food vacuoles. Bearing in mind their probable enzymatic nature, it is concluded that this

represents a process of intracellular digestion, similar to the process of extracellular digestion that occurs in gland cells, the secreted enzymes similarly arising in gland cells from the filamentous mitochondria.

I wish to take this opportunity of thanking Dr. O. W. Tiegs, of Melbourne University, for his valuable help and suggestions.

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THE FUNCTION OF THE LIPOID IN MITOCHONDRIA

by

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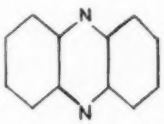
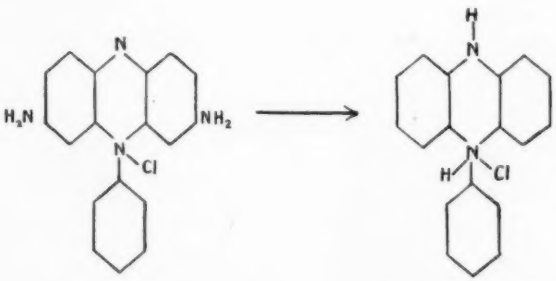
It has been shown by H. R. Marston (7) that the proteolytic enzymes form sparingly soluble compounds with dyes of the azine series, that is, with those dyes which contain the group:



The proteolytic substances are precipitated completely from crude enzyme solutions by the addition of soluble azines at suitable hydrogen ion concentrations. The point of union of the enzyme and the dye is indicated sufficiently clearly by the fact that the reduced "leuco" derivatives are no longer able to precipitate these enzymes:



Dye (red). Reduced leuco compound.

It is therefore the basic nitrogen of the azine nucleus which is responsible for the precipitation.

It is well known that certain azine dyes, such as Janus green, are specific staining agents for mitochondria. It is, in fact, through the employment of these dyes that the universal distribution of mitochondria in living, nucleated cells has been established (2). It has been suggested by Marston that the specific

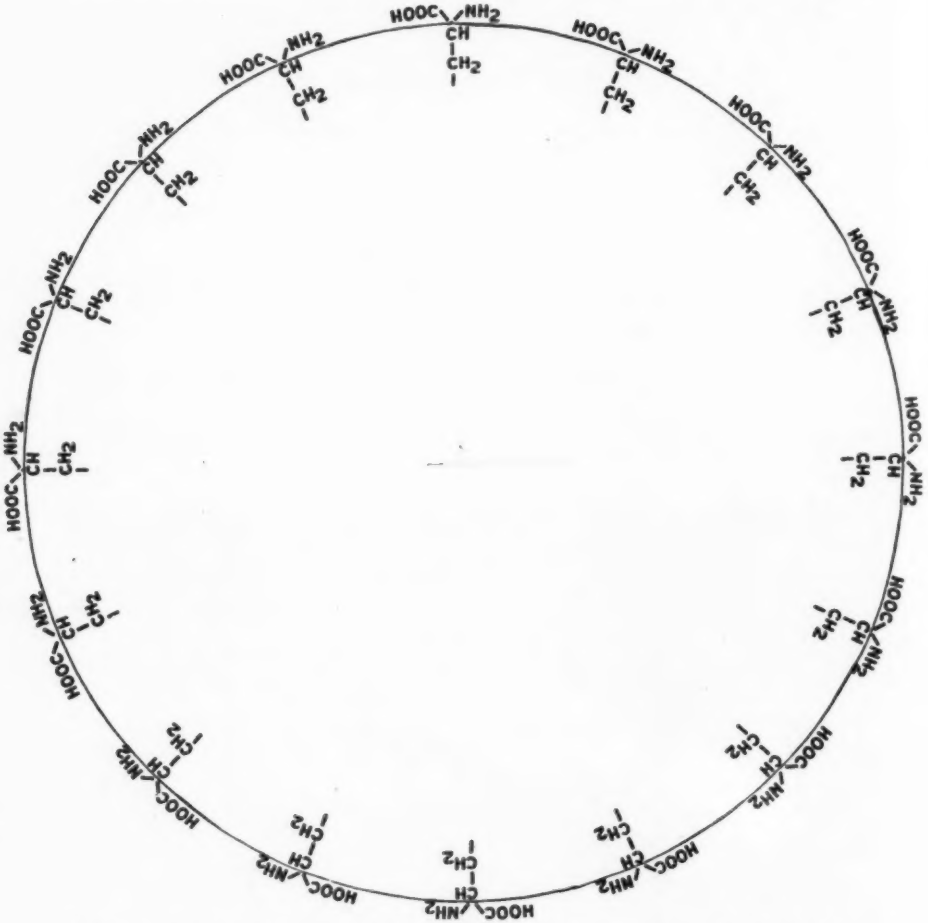


Figure 1. Illustrating the orientation of amino acids at the surfaces of mitochondria.

staining of mitochondria with azine dyestuffs may indicate that the intracellular proteolytic enzymes are concentrated in these bodies. "The mitochondria may thus be the site of syntheses in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favourable conditions for the synthetic activities of the enzyme."

The lipoidal character of mitochondria is indicated by their solubility in alcohol, ether, chloroform, and other lipoid solvents, and by the fact that they frequently blacken when treated with osmic acid. Concentration of amino acids or other soluble products of digestion at the surface of a non-aqueous phase, in conjunction with synthesizing enzymes, would in itself afford a favourable condition for their re-synthesis into the colloidal constituents of the cell, but it is probable that in the synthesis of proteins, and possibly also of fats and polysaccharides, the lipoid of the mitochondria facilitates synthesis in another way as well, namely, by so orienting the molecules of the hydrolysate as to accomplish a concentration of reactive groups which would not otherwise be attainable.

The amino acids, with the exception of the heterocyclic acids, histidine, proline, oxyproline, and tryptophane, and the diamino acids, arginine and lysine, consist of the active groups, —COOH and —NH_2, attached to hydrocarbon chains of varying length. From the work of Langmuir on the orientation of fatty acid molecules at the surface of aqueous phases (6), and that of Harkins upon the orientation of molecules containing other active groups, such as the amino group itself (4), it appears that at the surface of a lipoidal phase the —COOH and —NH_2 groups of amino acids must be oriented towards the aqueous phase, while the lyotrope hydrocarbon radicles will be oriented towards and, probably, immersed within the lipoidal phase. Through the imposition of this orientation, which is diagrammatically illustrated in the accompanying figure, the movements of the molecules of hydrolysate must be greatly constrained, being confined to rotation around the axis constituted by the lyotrope carbon chain. Under any other conditions, even in the solid (non-crystalline) condition, the molecules must be oriented in every possible direction, and the mean distance apart of the reactive groups must be greater because every molecule may successively present any part of itself to the adjacent molecules. With all of the reactive groups oriented in one direction, and molecular motion constrained to rotation around the lyotrope axis, however, every molecule must present to adjacent molecules the one reactive group or the other, and the effective concentration of —COOH and —NH_2 groups must be immensely greater than that attainable by the mere removal of water from the reacting system.

It has been shown by Taylor (9), and the author (8), and latterly confirmed by Wasteneys and Borsook (11) that proteolytic enzymes may accomplish resynthesis of protein from amino acids, provided the enzyme is present in relatively high concentration. Concentration of the hydrolysate and elevation of temperature are favouring conditions for synthesis, but no concentration of the hydrolysate suffices to achieve complete resynthesis of amino acids into protein. The same has also been shown to be true in the synthesis of fats by lipase (1). So far as proteins are concerned, however, the low concentration of free amino groups in living tissues, and the high relative concentration of

proteins, and their stability, unite to indicate that notwithstanding the high proportion of water contained in living cells, synthesis of amino acids into protein is virtually complete.

It appears probable that this relative completeness of synthesis is brought about through the uniform orientation and restraint of motion of the molecules of hydrolysate by the lipoidal constituents of the mitochondria. If this be so, then the same mechanism affords an explanation of a fact which otherwise appears to be at present inexplicable, the fact, namely, that all of the amino acids in protein are α-amino acids[1], that is, the amino group stands in the closest possible proximity to the carboxyl group. We can readily understand how this configuration must conduce to the stability of the resultant protein by shortening the chains which unite the amino acids to one another, but this fact does not explain how it is that the protein manufactured by living cells comes to be of this stable type. The mechanism of synthesis which has been suggested, however, would render it inevitable that the amino acids which unite to form proteins must be α-amino acids, because only acids of this configuration could simultaneously expose both of their active groups to the surface of the aqueous phase which contains the enzyme.

The single apparent exception to the rule that amino acids derivable from proteins are α-amino acids is that afforded by lysine, and the behaviour of this amino acid, when it enters into combination with others to form protein, affords the most remarkable confirmation that could be desired of the above interpretation of the mechanism of protein synthesis within the cell. For although lysine possesses both an α- and ω-amino group, and the latter, owing to its distance from the carboxyl, is probably the more basic group[2] (5), yet in proteins it is the α-amino group which is combined with the carboxyls of other amino acids, while the w-amino group remains uncombined, and actually constitutes the only free amino group in the whole of the protein molecule (10). Evidently in the synthesis of a protein the α-amino group of lysine must be brought into close proximity to the carboxyls of other amino acids, while the ω-amino group must be prevented in some fashion from attaining proximity to the carboxyl group of any other acid. It is difficult to see how this could be achieved by any mechanism other than one which maintains such an orientation of the amino acid molecules during the process of synthesis, that the carboxyls and adjacent amino groups are contiguous to, and other portions of the molecules removed from the region of chemical interaction.

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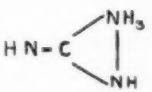
[1] Strictly, proline and oxyproline are not amino acids at all, but α-amino acids.

[2] On the other hand the slowness with which nitrous acid reacts with the ω-amino group of lysine (10) may indicate that this is the less active group of the two. Even if this be so, however, the weaker of the two amino groups in lysine is of about the same strength as the α-amino groups in aspartic and glutamic acids (5), and these are completely neutralized by carboxyl groups in proteins.

The inquiry may be raised why the ω-amino group of lysine, being an active group, does not also become oriented towards the water surface? It is not without significance in this connection that lysine contains the longest lyotrope carbon chain of any of the amino acids occurring in protein. We have at the one extremity of the chain two active hydrophil groups, at the other only one. Presumably the combined effects of the carboxyl and α-amino groups overbalance that of the single ω-amino group, and, because the chain is so long, oscillations of position would be difficult, the entire lyotrope effect of the intermediate chain of hydrocarbon groups having to be overcome before an inversion of orientation can be achieved. Similarly, in para derivatives of benzene, when the two groups in the para position are equally active the molecule lies upon the surface of water because both active groups cannot be simultaneously immersed, but if one group surpasses the other in activity it is drawn into the water, and the benzene ring is consequently tilted up on edge (4).

Arginine, as the structure of that molecule is ordinarily written, would appear to constitute another exception to the rule, but in fact it does not, for the work of Werner indicates that the guanidine derivatives, analogously to urea, are actually ring compounds (12). The structure of guanidine itself is probably:



so that if arginine is analogously built up it can only contain one amino group, namely, that which is in the α position. The absence in protein of any free amino groups corresponding to their arginine content is exactly what we should expect if the structure of arginine is analogous to that of guanidine and urea.

The α-amino groups in lysine are neutralized in proteins by carboxyl groups, and so are the α-amino groups in all of the other amino acids which participate in the construction of the protein. Just as many carboxyl groups must have been neutralized as amino groups, and since each amino acid has contributed at least one amino group for neutralization, each amino acid must also have contributed one carboxyl group. Only those carboxyls can remain free in the protein molecule, therefore, which are contributed by the second carboxyls of the dicarboxylic acids, glutamic acid, hydroxyglutamic acid, and aspartic acid. Proceeding upon the analogy afforded by lysine, we would infer that the free carboxyls must be those most distant from the amino groups, but unfortunately we lack as yet any means of distinguishing between the two carboxyls of a dicarboxylic acid in such a manner as to enable us to determine which of them remains unattached in the protein molecule.

So far as the amino acids derived from the aliphatic series are concerned, the theory herein presented is consistent with all of the known facts. It must be admitted, however, that data are as yet lacking which would enable us to predict the orientation at a lipoid-water surface of such molecules as those of histidine, tryptophane, and arginine. The fact that these also enter into the protein molecule through the agency of their α-amino groups, however, suggests that the combined activities of the carboxyl and α-amino groups overcome any affinity for the water phase which may be possessed by the cyclic portions of these molecules.

SUMMARY.

Following the suggestion of H. R. Marston, that the surfaces of the mitochondria are the loci of syntheses in the cell, it is pointed out that the lipoid composition of these bodies would tend to orient molecules at their surfaces in such a manner that the reactive groups, for example, the carboxyl and α-amino groups of amino acids, would all point towards the aqueous phase, while the lyotrope hydrocarbon chains would be buried in the lipoid phase. The movements of the molecules would thus be restrained and confined to rotation around the axis of the hydrocarbon chain, so that the reactive groups would attain an effective concentration, at the surface of the aqueous phase, which could not possibly be attained in any other manner. It is suggested that this affords an explanation of the completeness of enzymatic syntheses in living tissues, which has not hitherto proved attainable *in vitro* by mere concentration of the hydrolysate through removal of water from the reacting system. It is also pointed out that this mechanism of synthesis would necessitate the observed predominance of α-amino acids among the products of the hydrolysis of proteins, and would also render explicable the fact that lysine is united to the remainder of the protein molecule by its α-amino group, while the ω-amino group remains uncombined.

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THE PERMEABILITY OF POLARIZED MEMBRANES IN RELATION TO THE PERMEABILITY OF THE NUCLEUS AND THE PROPORTIONS OF THE VARIOUS AMINO-ACIDS WHICH ARE CONTAINED IN PROTEINS

by

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It has been shown by Macallum (15) that the nucleus of the living cell lacks certain inorganic constituents, chlorides, phosphates, and potassium, which are characteristic components of the surrounding cytoplasm. It appears extremely probable from the evidence which is at present available that inorganic constituents, in the uncombined condition at all events, are totally absent from the nucleus, for while certain inorganic substances, such as iron for example, and possibly manganese, appear to be present in nuclei, it is possible that they exist therein in combination with organic radicles, just as phosphoric acid is present in combination with carbohydrate and nitrogenous bases to form nucleic acid. At all events, the absence of chlorides speaks very strongly against any interchange of inorganic salts between nucleus and cytoplasm, and we must infer that the nuclear membrane is of such a nature as to be impermeable to the inorganic electrolytes which are present in the cytoplasm which surrounds it.

This being the case, the nucleus must either be subjected to and capable of withstanding the very considerable osmotic pressure (about 7 atmospheres) of the inorganic electrolytes contained in cytoplasm, or else some other dissolved substances must take the place of these and maintain an osmotic pressure within the nucleus which approximates to the osmotic pressure of the surrounding cytoplasm. On a similar principle to that involved in the Donnan equilibrium we would expect to find such substances relatively concentrated within the nucleus, just as the inorganic electrolytes are relatively concentrated without.

It has been shown by Van Slyke and Meyer (24) that following upon absorption of the products of protein hydrolysis from the intestine, the amino

nitrogen content of tissues increases up to a saturation limit which is characteristic for each tissue, and that thereafter the tissues become desaturated at varying rates. There is, therefore, a storehouse for amino acids within the cells, which is of somewhat variable capacity.

The possibility occurred to Collip (2) that the nucleus might form such a storehouse, the amino acids which it contains assisting to maintain osmotic equilibrium between its interior and its exterior. Accordingly he investigated the content of free animo groups in nucleated and non-nucleated cells which are as nearly as possible similar in other respects, the nucleated red corpuscles of birds, namely, and the non-nucleated corpuscles of the mammalia. He found the content of free amino groups far higher in the former, indicating the presence of far greater amounts of amino acids in nucleated cells than in non-nucleated cells of similar type and function. He concluded that the nucleus, among other functions, probably performs that of storing amino acids.

It must be admitted that this evidence, taken by itself, is not conclusive, for nucleated corpuscles differ from non-nucleated corpuscles in other particulars besides the possession of a nucleus. For example, nucleated erythrocytes contain mitochondria, while non-nucleated erythrocytes do not (3). Nevertheless, the properties and functions of the mitochondria, so far as we know or surmise them, do not suggest that they officiate as storehouses of amino acids, but rather as a means of converting them, when necessary, into protein (16). The mitochondria are composed very largely of lipoid materials, and amino acids are not lipoid-soluble substances (9). Amino acids could only be expected to accumulate at their surfaces, therefore, and in case they did so they would probably be converted into protein (22). There must, in fact, exist some method of preventing the access of excess of amino acids to the mitochondria, otherwise, it would appear, synthesis of protein would be the immediate response to ingestion of amino acids from the alimentary canal. This implies a means of storing the surplus amino acids in some other locality within the cell.

If, however, we accept the decision of Collip that the nucleus forms the storehouse of amino acids within the cell,[1] then certain curious implications are involved concerning the permeability of the nuclear membrane.

We have here a membrane which, while impermeable to ordinary electrolytes, its nevertheless permeable to amino acids, which are amphoteric electrolytes. The mere fact that it is penetrable by these substances would ensure their accumulation within the nucleus, because osmotic equilibrium is thereby maintained, but however great their relative concentration within the nucleus, this

[1] In certain unicellular forms (for example, Infusoria) the two functions of the nucleus, that of acting as storehouse for soluble food-reserves, and that of conveying hereditary qualities of the cell, are partially separated, and there are two nuclei, the micro- and the mega-nucleus, of which the latter has for long been generally supposed to officiate mainly as a storehouse of food-reserves.

would nevertheless be determined by the lower concentration of amino acids in the cytoplasm, so that diminution of the latter would lead to issuance of amino acids from the nucleus to replace the deficit, and conversely augmentation of the amino acid concentration in cytoplasm would lead to a proportionate augmentation of amino acid concentration within the nucleus, the forces determining entry or exit being osmotic.

What nature of membrane would permit the entry of amphoteric electrolytes, while interposing a barrier to the passage of electrolytes of a non-amphoteric character? The mere contrast of the permeabilities of these two classes of electrolytes suggests an electrical origin of the phenomenon, and, in fact, a membrane which is electrically polarized, might be expected to possess precisely the properties which Collip has inferred, are possessed by the nuclear membrane.

The effects of such a surface upon the orbits of approaching ions are illustrated in figure 1, in which the surface of the nucleus itself is represented as negatively charged, while that of the surrounding cytoplasm is represented as positively charged.[2] Any approaching positively charged ion would be repelled by the positive (induced) charge upon the cytoplasmic surface.[3] Any approaching negative charge would penetrate the positive layer but be repelled by the negative layer of charges. An amphoteric substance in solution, however, at any reaction in the neighbourhood of its isoelectric point, yields a proportion of doubly-charged, or "Zwitter-ions" (26). Such ions would be polarized by the electrical double layer itself, presenting their negative charges to the positively charged layer and their positive charges to the negatively charged layer. There would thus be nothing to prevent their penetration of or exit from a surface of this character. We may infer, therefore, that if Collip's evidence be taken as affording satisfactory proof that the nucleus acts as a storehouse for amino acids, then it must also be regarded as affording proof that the surface of the nucleus is electrically polarized.

The concentration of Zwitter ions in a solution of an amphoteric electrolyte rises rapidly as the reaction approaches the isoelectric point of the amino acid. From this it follows that the penetration of the nucleus by any particular amino acid will become much more difficult the further the P_H of the cytoplasm recedes from the isoelectric P_H of that amino acid. If all of the amino acids which gain entry into the circulating fluids and the tissues were presented to the surface of the nucleus in equal concentration, then that acid of which the isoelectric P_H approaches most closely to the P_H of the cytoplasm would accumulate more readily within the nucleus than any other. The concentration of any amino acid

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[2] The surface of the nucleus has a high oxidative potential (20, 25), and hence must contain an excess of negative charges. The retention of amino acids within the nucleus may thus depend upon the maintenance and magnitude of the oxidative potential at its surface.

[3] Or, which comes to the same thing, would take the place of one of the positive ions, which would, in turn, be repelled.

within the nucleus would in that event depend primarily upon the closeness of its isoelectric P_H to the actual P_H of the cell. The concentrations of acids having isoelectric points in this neighbourhood would be high; those having isoelectric points receding from this value on either side would be present in rapidly diminishing concentrations. The relative concentrations of amino acids stored within the nucleus, subsequently to become available, if required, for the synthesis of protein, would, therefore, in this event be proportional to some function of their Isoelectric P_H values, or:

$$c = F(I.P_H),$$

where c is the concentration of any particular amino acid and $F(I.P_H)$ is some undefined function of the Isoelectric P_H, $(I.P_H)$.

Actually, however, amino acids are not presented to the cell in equal, but, on the contrary, in very unequal proportions, and since the forces which necessitate the passage of amino acids across the polarized membrane at the surface of the nucleus are osmotic forces, the concentration in which they are presented to the cell or to its nucleus must participate in determining the concentration which is attained within the nucleus itself. In other terms:

$$c = C\,F(I.P_H),$$

where C is the concentration in which the particular amino acid is presented to the surface of the nucleus. From this it follows that:

$$\frac{c}{C} = F(I.P_H),$$

that is, the ratio of the concentration of the amino acids *within* the nucleus to their concentration *without* the nucleus should be some undetermined function of the isoelectric P_H values for the acids, and this ratio should exhibit a sharp peak in the neighbourhood of the P_H of the cytoplasm, which possibly differs slightly in the cells of different tissues.[4]

We are not in a position to evaluate directly either of the quantities c or C. But if the proportion in which the various amino acids are stored within the cell represents the proportion in which they are available for the synthesis of protein by that cell, and they are totally utilized, then the amino acid composition of the hydrolysate derived from any protein should reflect fairly closely the relative proportions of amino acids stored within the nuclei of the cells from which it was derived. This would be true if the available acids were totally utilized and if the mitochondria played no part in *selecting* the amino acids which are to be reconstituted into protein, but passively accept whatever is presented to them.[5] It is possible that some selection does actually occur, since Harkins has shown that the proportions of various molecules which concentrate at a fluid

[4] The observations of Harvey (7), Jacobs (11), and others demonstrate that the P_H of cell contents may not be directly inferred from that of the surrounding medium. It is usually more acid owing to accumulation of CO_2 within the cell (11).

[5] Cf. preceding paper.

surface are determined, not solely by their relative concentration in the body of the solution, and the reduction of surface energy which they severally bring about, but also by the conformations of the molecules and the ease with which they can be fitted together to form a monomolecular layer (5). Still, admitting the probability that these factors may play an important part in determining the amino acid composition of the proteins, we may still affirm that the *availability* of the several amino acids for synthesis into protein must be a factor in deciding the proportions of amino acids which will enter into that protein, and since the concentration of Zwitter ions in an ampholyte solution presents such a steep maximum in the neighbourhood of the isoelectric point,[6] we would confidently expect to see this peak reproduced in the composition of a protein if Collip's hypothesis of amino acid storage were correct.

The values of C are still more difficult to ascertain. We cannot take them to be proportional to the concentration of amino acids in the dietary, for we know from the results obtained by Abderhalden, London, and Oppler (1) that amino acids are not absorbed from the alimentary canal in the proportions in which they are presented to the animal in the diet. The more closely the dietary proteins approximate to the composition of the body proteins, the more completely are they absorbed, and the less they correspond to the composition of the body proteins of the animal the more resorting by the alimentary canal is required and the more wastage of unutilizable amino acids is involved. These excesses of amino acid are passed on unabsorbed, and in consequence the "biological value" of the protein falls (23). The relative concentrations of the amino acids in the blood are unknown, for at present the resources of our analytical technique are insufficient to ascertain them. From the above-cited results of Abderhalden, London, and Oppler, however, and our general familiarity with the economy of effort consequent upon adaptation which characterizes living organisms,[7] we may infer with some confidence that, selection having been performed by the alimentary canal, it is final, and that all that is passed on to the tissues is potentially utilizable for constructing protein. If this be the case, then the total amino acid composition of the organism should reflect the normal proportions of amino acids which are presented to it, some being utilized predominantly in one type of cell and to produce one type of protein, others in other types of cell to produce other types of proteins, the wastage in the construction of the whole animal being a negligible minimum.

The amino acid composition of the whole animal is unknown, but Osborne and Jones have determined the amino acid composition of the muscle tissue of the ox (19). This is not quite such a representative tissue as we could desire for our purpose, but still it is a mixture of many kinds of cells, and doubtless

[6] Similar to the maximum of flocculation.

[7] The biological equivalent of the principle of Le Chatalier.

represents a closer approximation to the composition of the body as a whole than to that of any particular protein which it contains.

Our knowledge of the amino acid composition of protein hydrolysates has recently become more accurate, owing to the discovery by Dakin of the method of extracting the mono-amino acids from the hydrolysate with butyl alcohol (4). The aggregate nitrogen of the amino acids thus determined amounts to over 90 per cent. of the total nitrogen in the protein, previous methods yielding aggregate values of only 60 or 70 per cent. Unfortunately, only three proteins have as yet been analyzed by this method, namely, gelatin (4), casein (4), and lactalbumin (12).

The analysis of muscle tissue by Osborne and Jones was made before this method had become available. Comparison of Dakin's analysis of gelatin with older analyses indicates that in Osborne and Jones' analysis alanine and some other amino acids may be deficient, while glutamic acid may be in excess. Still the analysis accounts for over 70 per cent. of the total nitrogen, and the errors, as we shall see, do not suffice to mask, although they doubtless modify, the effect for which we are seeking.

Dividing the concentrations of the amino acids found in the hydrolysate from any protein by the concentration of the corresponding acids in muscle tissue (regarded as a sample, admittedly defective, of the whole animal), this ratio plotted against the isoelectric P_H values of the amino acids concerned should, if Collip's hypothesis is correct, yield a curve presenting a peak at the P_H of the cell in which the protein was manufactured, and falling rapidly on either side of this peak.

The results of plotting these ratios for gelatin, casein, and lactalbumin against the isolectric P_H values of the respective amino acids are shown in the graphs illustrated in figures 2, 3, and 4. The estimates of isoelectric P_H which are employed in these graphs are enumerated in the accompanying table, together with the sources from which they are obtained:

TABLE OF ISOELECTRIC P_H VALUES FOR AMINO ACIDS.

(Calculated from the formula: $-2P_H = \log Ka + \log Kw - \log Kb$ where $\log Kw = -13{\cdot}90$ at 25°C.)

Amino Acid.	Isoelectric P_H.	Author.
Aspartic Acid	2·76	Levene and Simms (14)
Glumatic Acid	3.23	Harris (6)
Cystine	4·30	Merrill (18)
Phenylalanine	5·35	Kanitz (13)
Tyrosine	5·65	Hitchcock (8)
Valine	5·95	Harris (6)
Leucine	5·97	Harris (6)

Amino Acid.	Isoelectric P_H.	Author.
Glycine	6·03	Harris (6)
Alanine	6·17	Harris (6)
Proline	5·30	McCay and Schmidt (17)
Histidine	7·15	Kanitz (13)
Lysine	9·40	Kanitz (13)
Arginine	10·97	Hunter and Borsook (10)

The results bear out our anticipations in rather a remarkable fashion, considering the number of uncertainties and approximations which are involved in obtaining them. The peak in each curve is so very prominent that the effect of polarization at the surface of the locus of amino acid storage is instantly suggested. Moreover, the peak is in each case in the neighbourhood of the P_H which we would expect in the contents of a living cell, a P_H, that is, a little on the acid side of neutrality (7, 11). The crowding of amino acid isoelectric P_H values in this neighbourhood is also very significant, and suggests that in the synthesis of all proteins this factor plays an important rôle.

Other proteins have been plotted in a similar manner, and reveal similar sharp maxima in various regions, but when the uncertainties of the older analyses are superadded to the uncertainties of Osborne and Guest's analysis of muscle tissue, coupled with the poorly representative character of this tissue itself, the accumulation of errors may distort the curves so greatly that it has not been thought worth while to reproduce any others here. Some exhibit subsidiary maxima which may very easily have originated in the accumulation of analytical errors. In one case, however, we may be sure that the second maximum is real, namely, in the case of salmine, which contains 87 to 91 per cent. of arginine, no lysine or histidine, with 11 per cent of proline, and 4 to 5 per cent. of leucine. The circumstances under which the proteins in spermatozoa are synthesized, however, are so peculiar that one is not surprised to find their composition departing from the usual rule. Among other interfering factors we may mention the frequency of mitoses in spermatogenic tissue with dissolution of the nuclear membrane, and consequent scattering of amino acids through the cytoplasm, and temporary lapsing of selective storage.

The question may be raised whether our estimates of the isoelectric P_H values of amino acids, particularly those of glycine, valine, and leucine, are sufficiently accurate to assure us that they are distributed in the order represented in the graphs. Undoubtedly they are not. Thus if the isoelectric P_H value for leucine were *greater* than that for glycine, the lactalbumin curve would display two sharp contiguous maxima. If the isoelectric P_H value for leucine were *less* than that for valine, the curve for gelatin would display two maxima. Yet the estimated isoelectric P_H for leucine differs by only +0·02 from that for valine, and —0·06 from that for glycine. We are not entitled to suppose that these

estimates have attained such a degree of accuracy as to ensure the correctness of such fine gradations of isoelectric values, and the single maxima displayed in these curves may therefore be accidental in origin.

The internal evidence afforded by the curves themselves, however, shows that the maxima are not accidental in origin. Apart from glycine, valine, and leucine, the ascertained values of the isoelectric P_H for the remaining acids are certainly of sufficient accuracy to guarantee that they are placed in their correct order upon the axis of abscissae. In each curve the presence of a maximum in the neighbourhood of $P_H = 6$ is indicated by several points upon the curve. Thus in the case of casein, which exhibits a maximum at valine, there are two points upon the descending limb of the curve upon the acid side (tyrosine and phenylalanine), and there is one point upon the more steeply descending limb on the alkaline side (glycine). In the case of lactalbumin, which exhibits a maximum at leucine, there are three points on the descending limb of the curve upon the acid side (valine, tyrosine, and phenylalanine), and these points descend in order of their distance from the maximum. In the case of gelatine, which exhibits a maximum at glycine, there is a point (leucine) which lies exactly upon the almost straight descending limb which intersects the axis at valine (absent in gelatin), and upon the less steeply descending limb of the curve on the alkaline side there are three points which descend in the order of their distance from the maximum, namely, alanine, proline, and histidine. The reality of the maxima thus indicated can hardly be doubted, and moreover the crowding of amino acids in the neighbourhood of $P_H = 6$, as pointed out above, contributes evidence from another angle pointing to the importance of isoelectric P_H in determining the facility with which amino acids can be employed in the manufacture of protein. The fact is probably not without significance, also, that of the three protein curves depicted in the graphs, those two (casein and lactalbumin) which are derived from the same organ (not necessarily the same cells) exhibit curves of like form, with a steeply descending limb on the alkaline side of the maximum, while gelatine, derived from a widely differing tissue, exhibits a curve which departs widely from this type, and displays a steeper descent on the acid than on the alkaline side of the maximum. Whatever uncertainty attaches to the absolute magnitudes of the isoelectric P_H values for the different amino acids, therefore, it appears highly probable that they are seriated in the correct order in the diagrams, and that the maxima in the curves are real, and point to the existence of a polarized surface within the cell which determines the availability of amino acids for synthesis into protein.

SUMMARY.

It has been shown by Collip that nucleated erythrocytes contain far greater amounts of free amino groups than non-nucleated erythrocytes. He infers that

amino acids, among other soluble substances, take the place of mineral constituents, which are absent from the nucleus, in maintaining osmotic equilibrium between the nucleus and the surrounding cytoplasm. It is pointed out that as the nuclear membrane is permeable to amphoteric electrolytes, but not to non-amphoteric electrolytes, it must be electrically polarized.

The relative proportions of the amino acids which are contained in the three proteins (gelatin, casein, and lactalbumin), which have been completely analyzed, point also to the existence of a polarized surface within the cell which determines the availability of amino acids for synthesis into protein.

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